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TNT Biodegradation by Natural Microbial Assemblages at Estuarine Frontal Boundaries

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14. ABSTRACT

This limited scope project primarily focused on whether estuarine transition zones (e.g., fronts, convergences, salt wedges) are sites of enhanced 2,4,6, Trinitrotoluene (TNT) mineralization, bacterial metabolism and degradation of aromatic organic matter. Evidence was gathered to determine what biogeochemical features of these transition zones may contribute to such differences. Criteria for success of this investigation included measuring elevated rates of TNT mineralization and bacterial growth in mixing zones between water masses. We found evidence that these water mass interfaces are sites of enhanced bacterial metabolism and TNT mineralization when examining salt wedges, a frontal boundary, and mixing experiments between freshwater and marine end members. The major outcome of this SEED project was a demonstration that energetics, such as TNT, are readily degraded by natural bacterial populations in estuarine and coastal environments that receive substantial terrestrial runoff rich in natural aromatic organic matter.

15. SUBJECT TERMS

TNT	Biodegradation	Estuarine
Energetics	Coastal	Bacterial production
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LIST OF ACRONYMS

Ac:Ad: Ratio of Acid to Aldehyde Moieties
ASI: Air-Sea Interface
BIX: Biological Index
BTEX: Benzene, Toluene, Ethylbenzene and Xylene
C: Carbon
CDOM: Chromophoric Dissolved Organic Matter
CO_2: Carbon Dioxide
CTD: Conductivity-Temperature-Depth Water Sampling Device
CuO: Cupric Oxide
d^{-1}: Per Day
DNA: Deoxyribonucleic Acid
DOC: Dissolved Organic Carbon
DoD: Department of Defense
DOM: Dissolved Organic Matter
EEM: Excitation-Emission Matrix
EPA: Environmental Protection Agency
Fe: Iron
GC/FID: Gas Chromatograph/Flame Ionization Detector
GC/MS: Gas Chromatograph/Mass Spectometry
H_2SO_4: Sulfuric Acid
HIX: Humification Index
HMX: Octahydro-1,3,5,7-tetranitro-1,3,5,7-tetrazocine
kg^{-1}: Per Kilogram
L^{-1}: Per Liter
mCi: Millicurie
mL: Milliliter
MNA: Monitored Natural Attenuation
N: Nitrogen
NaOH: Sodium Hydroxide
nm: Nanometers
OM: Organic Matter
PAHs: Polycyclic Aromatic Hydrocarbons
PARAFAC: Parallel Factor Analyses
PCBs: Polychlorinated Biphenyls
POC: Particulate Organic Carbon
POM: Particulate Organic Matter
ppb: Parts Per Billion
PSU: Practical Salinity Units
QSE: Quinine Sulfate Equivalents
RDX: 1,3,5-Trinitroperhydro-1,3,5-triazine
R/V: Research Vessel
SON: Statement of Need
TCA: Trichloroacetic Acid
TCE: Trichloroethylene

TNT: 2,4,6,-Trinitrotoluene
UL-: Uniformly Labeled
USGS: United States Geological Survey
UXO: Unexploded Ordnance
μg: Micrograms
μL: Microliter

KEYWORDS

bacteria
bacterial production
biodegradation
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coastal
energetics
estuarine
Gulf of Mexico
Kahana Bay
marine
Mississippi River
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PAHs
salt wedge
tidal front
2,4,6-trinitrotoluene

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ABSTRACT

This limited scope project primarily focused on whether estuarine transition zones (*e.g.* fronts, convergences, salt wedges) are sites of enhanced 2,4,6,Trinitrotoluene (TNT) mineralization, bacterial metabolism and degradation of aromatic organic matter (*e.g.* petroleum hydrocarbons, lignin). Evidence was gathered to determine what biogeochemical features within these transition zones may contribute to such differences, and what cellular changes in the microbial assemblage may be indicative of these environments (*e.g.* organotolerance). Criteria for success of this investigation included measuring elevated rates of TNT mineralization and bacterial growth in mixing zones between water masses. Three coastal water surveys and mixing experiments between water mass end members were performed in 2011 to address these environmental topics: 1) Mississippi River/Gulf of Mexico (15-16 March; subtropical biome); 2) Charleston Harbor (25-26 June; temperate biome); and, 3) Kahana Bay (1 August; tropical biome). ^{14}C-TNT mineralization rates (TNT ^{14}C-ring carbon conversion to $^{14}CO_2$) were normalized to total heterotrophic carbon metabolism (^{3}H-leucine incorporation) to account for differences in overall bacterial growth rate and transformation rates of other terrestrial aromatic organic matter (*e.g.* CDOM, lignin, naphthalene, phenanthrene). We found evidence that these water mass interfaces are sites of enhanced bacterial metabolism and TNT mineralization when examining salt wedges (Gulf of Mexico, Charleston Harbor), a frontal boundary (Charleston Harbor), and mixing experiments between freshwater and marine end members (Gulf of Mexico, Charleston Harbor, Kahana Bay). Two to 10-fold increase in bacterial growth rates across relatively short distances (meters) of a salt wedge (vertical fronts in Gulf of Mexico and Charleston Harbor) and a river convergence (horizontal front, Charleston Harbor) drove a large variation in degradation potential across stratified water masses. Likewise, TNT and phenanthrene mineralization rates were highest on both sides of the river convergence (Ashley and Cooper Rivers). Experimentally mixing freshwater and marine end members (Kahana Bay) resulted in higher rates of bacterial production (+62%), phenanthrene mineralization (+68%) and TNT mineralization (+33%) though the effect on production was time dependent (possibly a response to osmotic shock). These findings support the hypothesis that both overall heterotrophic metabolism and TNT mineralization are enhanced at frontal boundaries. Measuring elevated TNT degradation rates amongst natural bacterial assemblages may reconcile the lack of detectable energetic concentrations in coastal sediment with their expected presence due to known long-term exposure to ordnance or surface runoff from a shore-side range. If TNT metabolism is related to overall bacterial growth on other aromatics, then a more extensive knowledge of environmental controls of natural aromatic biodegradation may be used to predict TNT fate in coastal ecosystems. The major outcome of this limited scope project was to demonstrate that energetics, such as TNT, are readily degraded by natural bacterial populations in estuarine and coastal environments that receive substantial terrestrial runoff rich in natural aromatic organic matter. By seasonally repeating these measurements and mixing experiments across other DoD-relevant sites and further identifying the specific areas associated with energetic degradation, we will increase the likelihood of public and regulatory acceptance of attenuation by natural bacterial assemblages as a viable risk reduction alternative.

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OBJECTIVE

The objective of this work was to develop lines of evidence that environmental conditions at frontal boundaries enhanced TNT metabolism by natural microbial assemblages. Criteria for success of this limited scope project included measuring elevated rates of TNT mineralization and bacterial growth in mixed water masses. In addition, TNT mineralization rates were normalized to total heterotrophic carbon metabolism to account for differences in overall growth rate and transformation rate of other terrestrial aromatic organic matter (OM). These values will define the lability of TNT in natural ecosystems relative to other naturally-present forms of aromatic OM (*e.g.* lignin, humics, PAHs). If TNT metabolism is related to overall bacterial growth and other aromatic compound degradation, then the large body of knowledge on environmental control of the latter may be used to predict fate of TNT in coastal ecosystems (see ER-SON-11-1.2: Assess the predominant fate and transport mechanisms and the kinetics of such reactions at environmentally relevant concentrations in both the water column and the aquatic sediments). By repeating these measurements and mixing experiments across other DoD-relevant ecosystems and further identifying the specific areas and conditions associated with energetic degradation, we will increase the likelihood of public and regulatory acceptance of natural attenuation as a viable risk reduction alternative. Documenting and validating such natural pollution abatement mechanisms supports continued use of active DoD ranges.

BACKGROUND

Our knowledge regarding fate, transport and ecological effects of energetic release lags far behind that of other organic (*e.g.* PAHs, PCBs, TCE, BTEX) and inorganic (*e.g.* metals, radionuclides) contaminants (see review by Brar et al. 2006). Over the past three decades, most of the research focus has been on terrestrial and groundwater systems (*e.g.* Army ranges) and applied laboratory work (*e.g.* flask biotreatability studies) rather than field ecology of estuarine and marine systems (Spain et al. 2000). As seen with virtually all contaminants, the application of laboratory studies to bioremediation at field sites has been problematic for both DoD and industry as laboratory cultured bacteria tend to poorly represent natural bacterial assemblages (Rappe' and Giovannoni 2001).

The magnitude of TNT source terms for active ranges and reported slow TNT degradation rate in terrestrial ecosystems suggests ranges have the potential to adversely impact adjacent coastal waterway ecosystems. Although the assumption has been that large historical input, coupled with bioaccumulation of energetics in tissues of fish and other higher organisms, poses a significant health risk to humans and associated biota, the actual supporting evidence for this is limited (Kilian et al. 2001, see review by Lewis et al. 2004). In the laboratory, toxicity studies with marine organisms have proven difficult to perform as TNT concentrations rapidly decrease in incubation chamber seawater over the course typical for such study (*e.g.* 28 days; Conder 2002) and much of the toxicity may be associated with the reduced products of TNT rather than the parent compound (see review by Roldan et al. 2008). Such studies with 1,3,5-Trinitroperhydro-1,3,5-triazine (RDX) are confounded by use of formaldehyde in preparing this

energetic (Ampleman et al. 2000) which complicates data interpretation in both toxicity and biodegradation studies (especially at higher energetic concentration). Field collection and measurement of energetics in marine organisms also suffer from lack of standardized methods for tissue extraction (*e.g.* sediment methods often used) and use of energetic detection methods prone to false positives (*e.g.* overlapping peaks with GC/FID for fish tissue in Pearl Harbor risk assessments).

In situ evidence supporting long residence time for energetics in coastal environments is also limited. Some of this lack of evidence has to do with the paucity of data collected from these environments. Though even when energetic analyses are performed on field samples, significant concentrations are rarely found in coastal ranges (*e.g.* Vieques Island; CH2M HILL 2006, Simmons et al. 2007) or offshore dumpsites (Oahu; Montgomery et al. 2008). This indirectly suggests that Monitored Natural Attenuation may be a viable remedial alternative for sites with recent or ongoing sources and No Further Action for those that were historically impacted.

TNT is often rapidly transformed and degraded by a variety of abiotic (*e.g.* photolysis, chemical hydrolysis, gamma irradiation) and biologically mediated processes (*e.g.* bacteria, microalgae, fungi, plants) (*c.f.* Spain et al. 2000). Though rates depend on environment and conditions, in general, rates of photolysis exceed microbial metabolism which exceeds chemical hydrolysis for TNT under surface water conditions common to coastal ecosystems (Montgomery et al. 2008). With the rapid extinction of light energy in surface water, microbial metabolism would be expected to be the predominant removal process for TNT with depth in the coastal water column and in most underlying sediment. Enormous variation in relative depths of photolysis vs. other process rates is expected in coastal ecosystems dominated by riverine inflow of OM and particulates. Depending on transformation process and physical conditions, TNT can be reduced to partial degradation products (*e.g.* aminotoluenes), mineralized to CO_2, incorporated into microbial biomass, or abiotically bound to non-living OM (*e.g.* humified; see review by Hawari et al. 2000). Virtually all of what is known about biological transformation of TNT is based on laboratory culture work using soil, freshwater, or groundwater isolates of eubacteria, cyanobacteria or fungi (see review in Spain et al. 2000).

Recently, there have been some reports using natural microbial assemblages from freshwater systems (Clark and Boopathy 2007, Douglas et al. 2009, Travis et al. 2008, Zheng et al. 2009), but outside of SERDP ER-1431, there is little work on TNT metabolism by natural estuarine or marine assemblages (Montgomery et al. 2008, 2009, 2011). In addition, despite numerous attempts with soil or freshwater samples, there are few reports of bacterial isolates that mineralize significant (*i.e.* >2%) amounts of TNT added to a batch culture. This had led to the widely held belief that the chemical structure of TNT makes the aromatic ring unavailable to attack by bacterial enzymes (Hawari et al. 2000) and that aminotoluenes produced by biological reduction, then bind to humic material rather than become incorporated into bacterial biomass (Myers et al. 1998, Thorn et al. 2002). However, Gallagher et al. (2010) have recently reported that TNT is metabolized by components of the estuarine bacterial assemblage (Elizabeth River sediment from the lower Chesapeake Bay) and that both TNT carbon and nitrogen are incorporated into bacterial nucleic acids (based on ^{13}C-TNT and ^{15}N-TNT using the Stable Isotope Probing method).

As part of SERDP ER-1431 (Montgomery et al. 2008, 2011), we found measurable TNT mineralization rates to CO_2 from natural microbial assemblages taken from several coastal ecosystems unlikely to have a history of exposure to energetics. During 15 sampling events in coastal waterways from 2002 to 2010, we measured TNT mineralization rates in surface sediment and water samples that were often the same as, or within one order of magnitude of, the rate of total heterotrophic bacterial metabolism. These rates were often similar to those of other organic compounds that are transient in natural ecosystems due to metabolism by bacteria, such as petroleum hydrocarbons and amino acids (Boyd et al. 2008). At first, these findings appear to conflict with those interpretations widely reported in the literature. However, our rates are very similar to many of those reports once their literature values are normalized to rate measurement units more typical of ecological studies of organic carbon metabolism ($\mu g\ C\ L^{-1}\ d^{-1}$ for aqueous samples, Appendix A; or $\mu g\ C\ kg^{-1}\ d^{-1}$ for sediment, Appendix B).

Growth of natural bacterial assemblages in freshwater and terrestrial ecosystems is typically phosphorus limited, while that of marine and estuarine assemblages is typically nitrogen limited (Pomeroy 1970, Paerl and Piehler 2008). Due to the metabolic cost of organic molecules synthesis, organic nitrogen sources are incorporated into other marine bacterial macromolecules (*e.g.* proteins, DNA) in preference to inorganic nitrogen species (Kirchman 1994 and references herein). Though it would be expected that an organic source of nitrogen would be incorporated into bacterial biomass, these rates for TNT are rarely reported in the literature. Some of this under reporting may have to do with the lack of recognition (in the bioremediation community) that bacterial metabolism of organic contaminants would be expected to involve the conversion of contaminant carbon to bacterial biomass. This may be because their focus is on complete detoxification of organic contaminant to harmless CO_2 rather than the metabolic fate of contaminant carbon. Instead of being recognized as incorporated, this fraction is often reported as being associated with humic material (*i.e.* humified), as ^{14}C-labelled organic compounds incorporated into bacterial macromolecules would typically co-precipitate in this analytical fraction (*c.f.* Nam et al. 1998).

Incorporation rates of ^{14}C-TNT into macromolecules of microbial biomass were measured in coastal water samples using the TCA precipitation method (Kirchman et al. 1985) and found to be one to two orders of magnitude more rapid than that for TNT mineralization (Montgomery et al. 2008). More specifically, 80-99% of ^{14}C-TNT that is fully metabolized by the microbial assemblage is incorporated into biomass rather than being respired for energy (as is typical for many organic sources of nitrogen in the marine environment; Montgomery et al. 2008). Incorporation rates and efficiencies were often highest at low salinity and then decreased towards the marine stations though occasionally the highest were at mid salinity (*i.e.* PSU = 10 at a frontal boundary; Montgomery et al. 2008).

These elevated mineralization and incorporation rates may be attributable to lignolytic marine fungi, rather than bacteria, though there is little precedence for fungi outcompeting bacteria for OM in marine environments. Lignolytic bacteria comprise a large component of the marine assemblage, for instance, the *Roseobacter* clade accounts for upwards of 20% of the natural bacterial assemblage in coastal waters with 16 of 19 identified strains harboring the gene for a key ring cleaving enzyme in lignin degradation (Gonzalez and Moran 1997, Buchan et al. 2000, 2001, 2005). In addition to their abundance, it is well established that bacteria are the

predominant degraders of lignocellulosic detritus in marine ecosystems (Benner et al. 1984, 1986). This suggests that our measured rates for TNT metabolism in coastal environments are most likely due to the bacterial component of the natural assemblage, rather than fungal. Although TNT mineralization by bacterial monocultures is seldom reported, the well-documented connection between lignin degradation and TNT mineralization (see review by Hawari et al. 2000) along with the predominance of bacteria as primary lignin degraders in estuarine systems (Benner et al. 1984, 1986) establishes the mechanism for TNT mineralization and incorporation by these natural bacterial assemblages. The recent finding of Gallagher et al. (2010) that TNT carbon and nitrogen are incorporated into bacterial DNA is further evidence that TNT is fully metabolized by components of natural assemblages in coastal ecosystems. It follows that in areas of the ecosystem where lignin is rapidly metabolized by the microbial assemblage, TNT may also be rapidly metabolized.

Prior work by our group showed evidence of this relationship between TNT and lignin degradation from examining sediment of the Chesapeake Bay system. In addition to TNT, microbial lignin degraders are known to metabolize and detoxify a wide variety of aromatic contaminants, such as PAHs and pesticides (*c.f.* Peng et al. 2008). The Patuxent River mouth appears to be a catchment for agricultural runoff including lignocellulose and nitrogenous organic pesticides, such as simazine (Harman-Fetcho et al. 1999, McConnell et al. 2004 and references therein). Terrestrial lignin will often migrate downstream to intermediate salinities and degrade only after the more labile organic fractions have been microbially metabolized upstream (Louchouarn et al. 1997). Sediment here is also associated with bacterial assemblages that rapidly metabolize such nitrogenous pesticides (McConnell et al. 2004). As part of our Chesapeake Bay system surveys in 2004 and 2005, we found sediment at this Patuxent River location (salinity = 12 PSU) to have the highest rates of TNT mineralization relative to total heterotrophic bacterial production (one to two orders of magnitude higher than all but one other river mouth station (Rappahannock; Montgomery et al. 2011)). *These transitional areas between freshwater and marine systems may trap otherwise recalcitrant aromatic materials (*e.g. *lignin, TNT, PAHs, pesticides) and select for assemblages that can rapidly metabolize these compounds.*

Transition zones between water masses can enhance organic metabolism by providing steady supply of nutrients and rapidly removing built up waste products. One side of the front can provide OM, nutrients (*e.g.* nitrogen, iron) or conditions (*e.g.* light, temperature) that are limiting to microbial growth on the other side (Floodgate et al. 1981). They can also inhibit lateral transport of organic particles and sediment which traps these materials and deposits them to the underlying sediment (Neill 2009 and references therein). These transition or mixing zones enhance production of bacteria (Borsheim 1990) and phytoplankton (Hyun and Kim 2003), remineralize nutrients via zooplankton grazing (Floodgate et al. 1981), and cycle nitrogen (Fogg et al. 1985). Not only are these fronts important regulators of OM processing in the water column, but tidal fronts moving back and forth can strongly influence and enhance OM processing in sediment underlying the passing front (Josefson and Conley 1997). By these mechanisms, these transition zones create biogeochemical conditions that increase overall heterotrophic bacterial metabolism and, by extension, may increase TNT metabolism.

On the macroscale, transition zones typically occur between water masses stratified by density differences due to salinity and/or temperature and include tidal fronts (vertically stratified; *e.g.*

salt wedges) and zones of convergence between rivers and higher salinity estuarine water (horizontally stratified; *e.g.* Gulf Stream rings). On the microscale that may be equally as relevant to biogeochemical functioning of the natural microbial assemblage, these transition zones will occur wherever there is input of freshwater or rain runoff into estuarine or marine coastal ecosystems. As such, at some level, these transitions zones may be characteristic of any DoD range or unexploded ordnance (UXO) impacted area adjacent to an estuarine or marine ecosystem. Specific examples of where freshwater creeks and rainfall runoff (0 PSU salinity) flow into adjacent estuarine or marine systems include Jackson Park to Ostrich Bay, WA (14-31 PSU; Carr et al. 2001), Moffett Field marsh to lower San Francisco Bay, CA (14-33 PSU; USGS 2007), Concord Naval Weapons Station to Suisun Bay, CA (mixing area between freshwater and seawater; Gross et al. 1999), and Live Impact Area of VNTR to BahAa Salina del Sur, Vieques, Puerto Rico (33 PSU; CH2MHill 2005).

In our previous SERDP work (Montgomery et al. 2008), we found that rates of TNT incorporation into microbial biomass and mineralization to CO_2 might be rapid enough to account for loss of range source material across the salinity gradient in estuarine systems (these rates were also used in an estuarine energetic attenuation model; Wang et al. 2009). There appeared to be some relationship between microbial TNT metabolism and salinity but the trend was not universal amongst the ecosystems studied for all times of the year. This may be because aromatic OM, like TNT, may be most rapidly metabolized at frontal boundaries that occur between water masses along the estuarine gradient (*e.g.* tidal fronts, salt wedges, and zones of confluence) and not a simple function of salinity. These frontal boundaries can be spatially narrow areas and may only randomly fall into the typical survey of an estuarine salinity gradient (or avoided entirely as being atypical of surrounding water). Variability in seasonal OM quality and quantity may also affect rates of TNT metabolism in estuarine gradients, but little is known about the interdependence of TNT metabolism, OM quality, and salinity.

This work compared rates of TNT metabolism by natural microbial assemblages at frontal boundaries between water masses and in underlying sediment in coastal ecosystems. The working hypothesis was that bacterial TNT metabolism would correlate with increased degradation of terrestrial aromatic OM and heterotrophic production. This relationship could help reconcile the lack of elevated energetic concentrations in coastal sediment with their expected presence due to known long-term exposure to ordnance or surface runoff from a shore side range. It could also reduce the calculated ecological risk associated with coastal sediments due to their association with UXO, obviating the need for energetic compound cleanup.

The primary technical challenge of this project involves sampling frontal systems at the temporal and spatial resolution needed to correlate relatively large-scale biogeochemical measurements with bacterial processes that occur on much smaller scales and change over unknown periods. Also concerning the temporal scale of changes at fronts, it may be challenging to determine the residence time of water masses that appear to be intermediate salinity along the frontal boundary. This latter feature may result in deviation between results obtained from interfacial boundaries in the field and mixing experiments using end member water masses. The most cost effective way to overcome these issues may be through iterative sampling of the relevant ecosystems over a variety of biome types to generalize our findings to range ecosystems that may have limited accessibility.

MATERIAL AND METHODS

Site Description One sampling was performed in each of three different ecosystems where we had experience in sampling and logistics and could cost-share ship time: Mississippi River/Gulf of Mexico; Charleston Harbor estuary; and Kahana Bay, Oahu. The Mississippi River/Gulf of Mexico site has a relatively high river flow, moderate OM concentration, and could provide laterally stratified confluences and mixing areas (NRC, 2008). The full salinity gradient (0-36 PSU) was sampled though vertically stratified gradients were only found outside the mouth of the Mississippi River (due to unusually high river flow prior to the sampling event). The second site, Charleston Harbor estuary, includes the Cooper River adjacent to the former Charleston Navy Yard and Westvaco Paper Mill. The Cooper River has a low net flow most times of the year and a well-defined salt wedge (Van Dolah et al. 1990). OM effluent (high in lignin content) flows from the paper mill into the Cooper and flocculates out along the salt wedge (vertically stratified 1-3 m below the river surface at the former Navy Yard). Sediment underlying the salt wedge in the Cooper River by the former Navy Yard contains bacterial assemblages with elevated PAH mineralization (likely due to lignolytic bacteria associated with the pulp mill effluent) (Boyd et al. 2008). In addition to the persistent salt wedge, a horizontally stratified front occurs during ebb tide at the confluence of the Ashley and Cooper Rivers in Charleston Harbor. The third site, Kahana Bay, is a tropical ecosystem (low OM concentration) with a defined and persistent salinity gradient from the fresh/brackish Kahana River to the open ocean over a relatively short transect (a few hundred meters) (Maciolek and Timbol 1981). Dramatic differences in river flow (as monitored by a USGS station in the river) affect the freshwater end member salinity and location of the mixing area. During this sampling, the river outflow (5 PSU) mixed with estuarine water over a shallow (0.5-1 m deep) shoal extending from the Kahana River mouth into the bay.

Sampling Sample locations for the Gulf of Mexico (Figure 1), Charleston Harbor Estuary (Figure 2) and Kahana Bay (Figure 3), were generally based on our previous sampling locations (Boyd et al. 2008, Montgomery et al. 2010, 2011). Water column (using Conductivity-Temperature-Depth (CTD) rosette with Niskin bottles), nepheloid samples (for nepheloid sampling devices see Pohlman et al. 2002, Appendix C) and sediment (multicorer) were collected along the salinity gradient of estuaries with sampling concentrated across salinity fronts between water masses and salt wedges. Depth profiles for salinity (PSU), dissolved oxygen concentration (DO, mL L^{-1}), and temperature (^{o}C) were measured and processed using ship deployed CTD rosette with sensors and Seabird® software for Gulf of Mexico (Appendix D) and Charleston Harbor (Appendix E). Kahana Bay surface water samples were collected by hand when wading from shore. Salinity of these samples was measured with a hand-held refractometer.

Cruise Schedule Mississippi River/Gulf of Mexico sampling was performed 15-16 March 2011 aboard the R/V *Pelican*. Charleston Harbor sampling was performed 25-26 June 2011 aboard the R/V *Cape Hatteras*. Kahana Bay sampling of the salinity gradient and frontal boundary was performed 1 August 2011 from the shore. Also included is a Kahana Bay sampling (20 July

2010) that was performed after the proposal was submitted but prior to start of project ER-2124 (Figure 4).

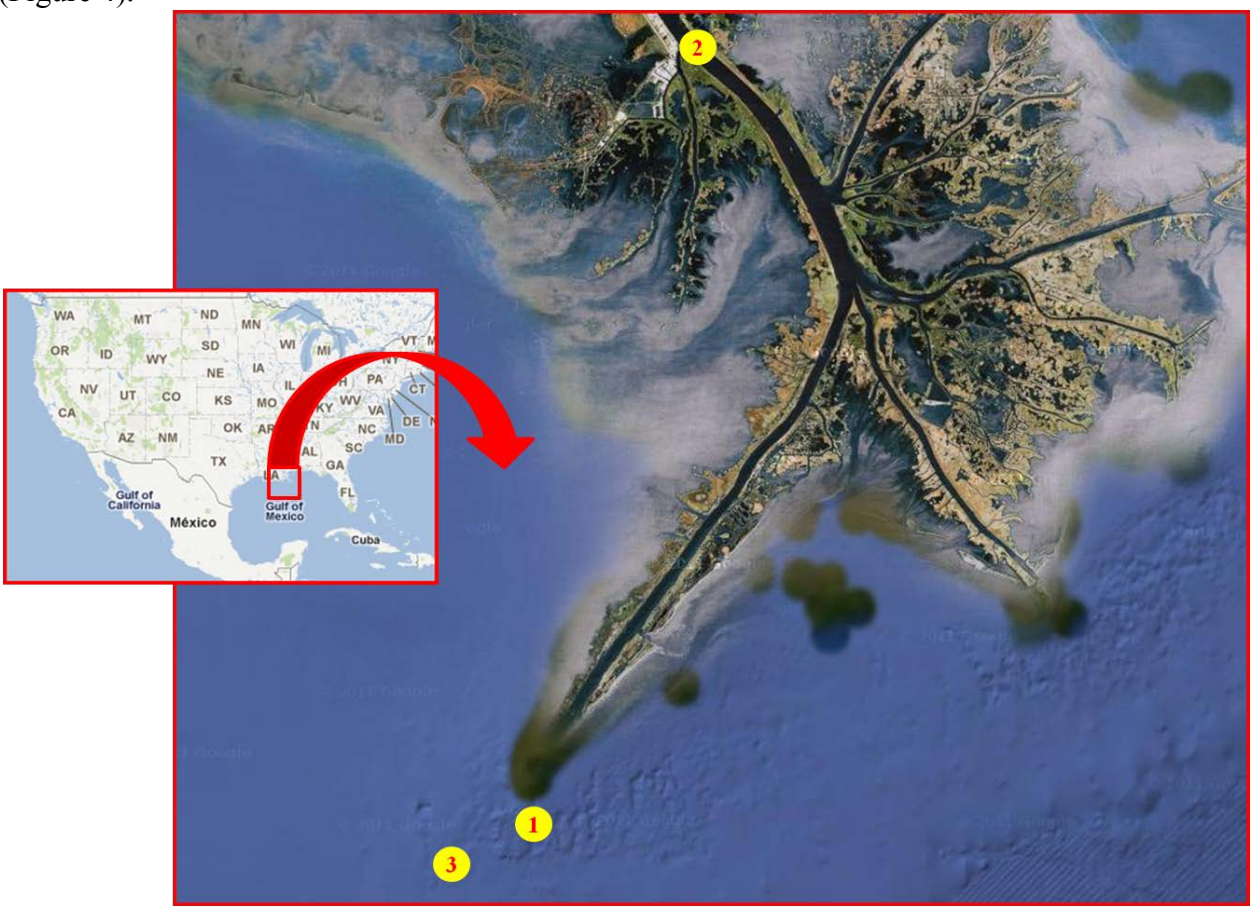

Figure 1. Sampling stations in the Mississippi River (MSY-2) and for salt wedge profiles (MSY-1, -3) in Gulf of Mexico off Louisiana, USA (15-16 March 2011).

TNT, Naphthalene, and Phenanthrene Mineralization Rates of bacterial metabolism of aromatic contaminants were measured by mineralization of ^{14}C-radiolabelled substrates to $^{14}CO_2$. These assays were initiated within two hours of sediment sample collection using a modification of Boyd et al. (1996) and Pohlman et al. (2002). 2,4,6-TNT-[ring-^{14}C(U)] (specific activity: 5 mCi mmol^{-1}; American Radiochemical Corporation, St. Louis, MO), naphthalene-[UL-^{14}C] (20 mCi mmol^{-1}), and phenanthrene-[9-^{14}C] (55 mCi mmol^{-1}) were added in separate incubations to surface sediment (1 mL wet volume) or 5 mL of seawater in 100×16 mm test tubes to a final concentration of about 0.2 μg g^{-1} or 0.04 μg mL^{-1} (depending on specific activity). For sediment, 0.5 mL of bottom water from the same station was filtered (0.22 μm nom. pore dia., Nuclepore polycarbonate) and added to make slurries with sediment that was cored from surface of the core section using a five-mL syringe with the end cut-off. Triplicate live and one kill (2 mL of 2 N H_2SO_4) of all samples were incubated for 48 h at *in situ* temperature in the dark and evolved $^{14}CO_2$ captured on NaOH-soaked filter papers. H_2SO_4 (2 mL, 2 N) was likewise added to end live incubations and to partition any remaining CO_2 into headspace of the tube and to the filter paper trap. Filter paper traps containing metabolized $^{14}CO_2$ were removed, radioassayed and subsequently used to calculate substrate mineralization. Triplicate one-mL syringed samples of

wet sediment were dried (overnight; 50°C) and used to convert mineralization values sample into kg sediment dry weight. Detection limit of the assay was typically 0.01 µg C L^{-1} d^{-1} though average values that were below one standard deviation were considered non-detect (0).

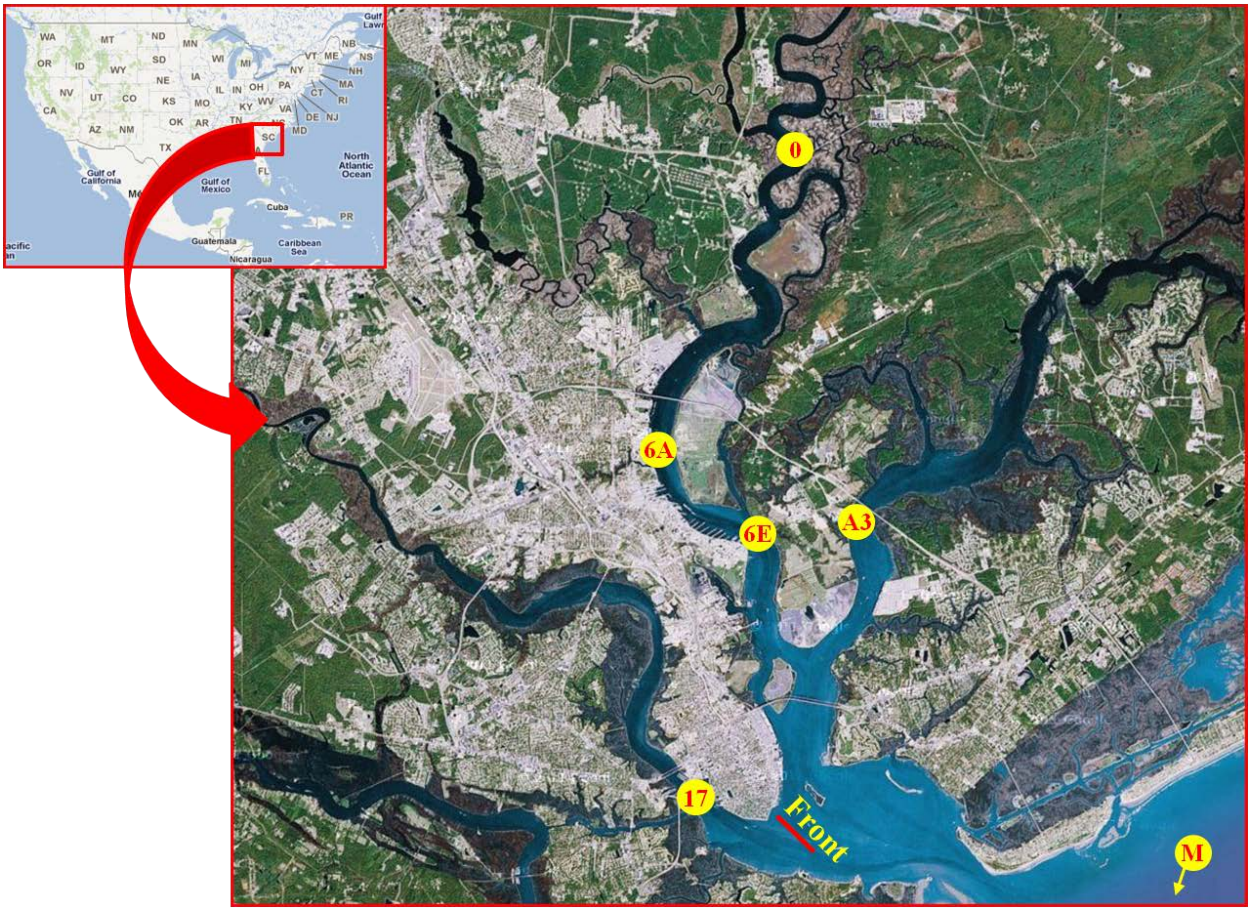

Figure 2. Sampling stations in Charleston Harbor, SC, USA (Front) and its major tributaries, the Cooper (0, 6A, 6E), Ashley (17) and Wando Rivers (A3), and offshore (M) on (25-26 June 2011).

Bacterial Production Growth rate of the heterotrophic bacterial assemblage (in terms of carbon) was measured by the leucine incorporation method of Smith and Azam (1992) for water or nepheloid (1.0 mL) or wet sediment (50 µL) as adapted by Montgomery et al. (2010). Environmental samples from each station were added to 2.0 mL microcentrifuge tubes (three experimental and one killed control) sealed with a cap with an O-ring that was pre-charged with [^{3}H-4,5]-L-leucine (120 mCi $mmol^{-1}$, final concn. 20 nM). Sediment was extracted from the sample using a one-mL polypropylene syringe with the end cut off and then added to the assay tube. For sediment, 1.0 mL of 0.22 µm (nom. pore dia.) filtered bottom water was then added to each tube and vortexed to form a sediment slurry. All samples were incubated at *in situ* temperature for 30 min. Incubations were ended by adding 57 µL of 100 % trichloroacetic acid (5 % final concentration; TCA, Fisher Scientific) and frozen for storage prior to processing by the method of Smith and Azam (1992). Killed controls have the TCA added prior to sample addition and these values were subtracted from those of the experimental samples. A constant isotope dilution factor of two was used for all samples and was estimated from sediment

dissolved free amino acids measurements (Burdige and Martens 1990) and saturation experiments (Tuominen 1995). Triplicate one-mL syringed samples of wet sediment were dried (overnight; 50°C) and used to convert sediment production values into dry weight. Leucine incorporation rate was converted to bacterial carbon using the formula of Simon and Azam (1989). Assay detection limit was 1.0 µg C kg^{-1} d^{-1} though average values that were below one standard deviation were considered below detection.

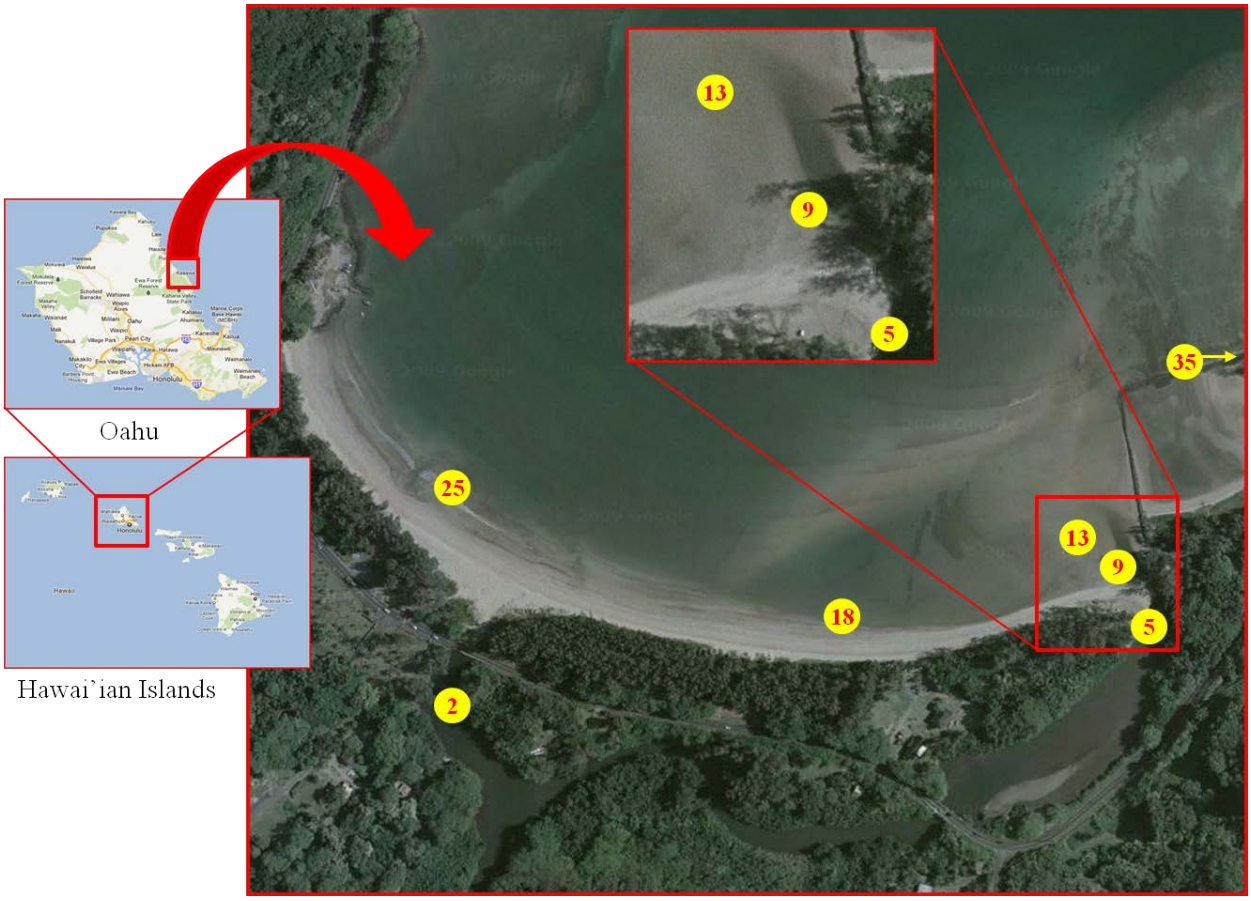

Figure 3. Sampling stations for a salinity transect (1 August 2011) from the Kahana River (2, 5) and Bay (9, 13, 18, 25), Oahu, HI, USA and Pacific Ocean (35). Station designations refer to their salinity (PSU). Shoal where river water mixes with estuary (inset).

Bacterial Organotolerance Inhibition of bacterial production by the presence of naphthalene (organotolerance) was measured as a proxy for osmotic stress at salinity fronts. Naphthalene organotolerance of the bacterial assemblage was measured by adding 0, 5, 15 or 25 µg of naphthalene dissolved in 5 µL of methanol to 0.50 µL of wet sediment or 1.0 mL of water and subsequently processed for bacterial production (Montgomery et al. 2010). All treatments and controls received the same addition of methanol (5 µL) though previous experiments showed that production was not affected in parallel incubations with methanol alone (Montgomery et al. 2010). The 5 µL of methanol with dissolved naphthalene was added to the microcentrifuge tube prior to sample addition. Average and standard deviation of three live incubations (with value for killed control subtracted) was regressed to the amount of naphthalene added to the leucine

incorporation assay. Final concentration of naphthalene in the 25 µg addition was ca. 250 µg g^{-1} sediment (dry weight) and 25 µg mL^{-1} of water. The regression formula and r^2 value were calculated using Microsoft Excel®.

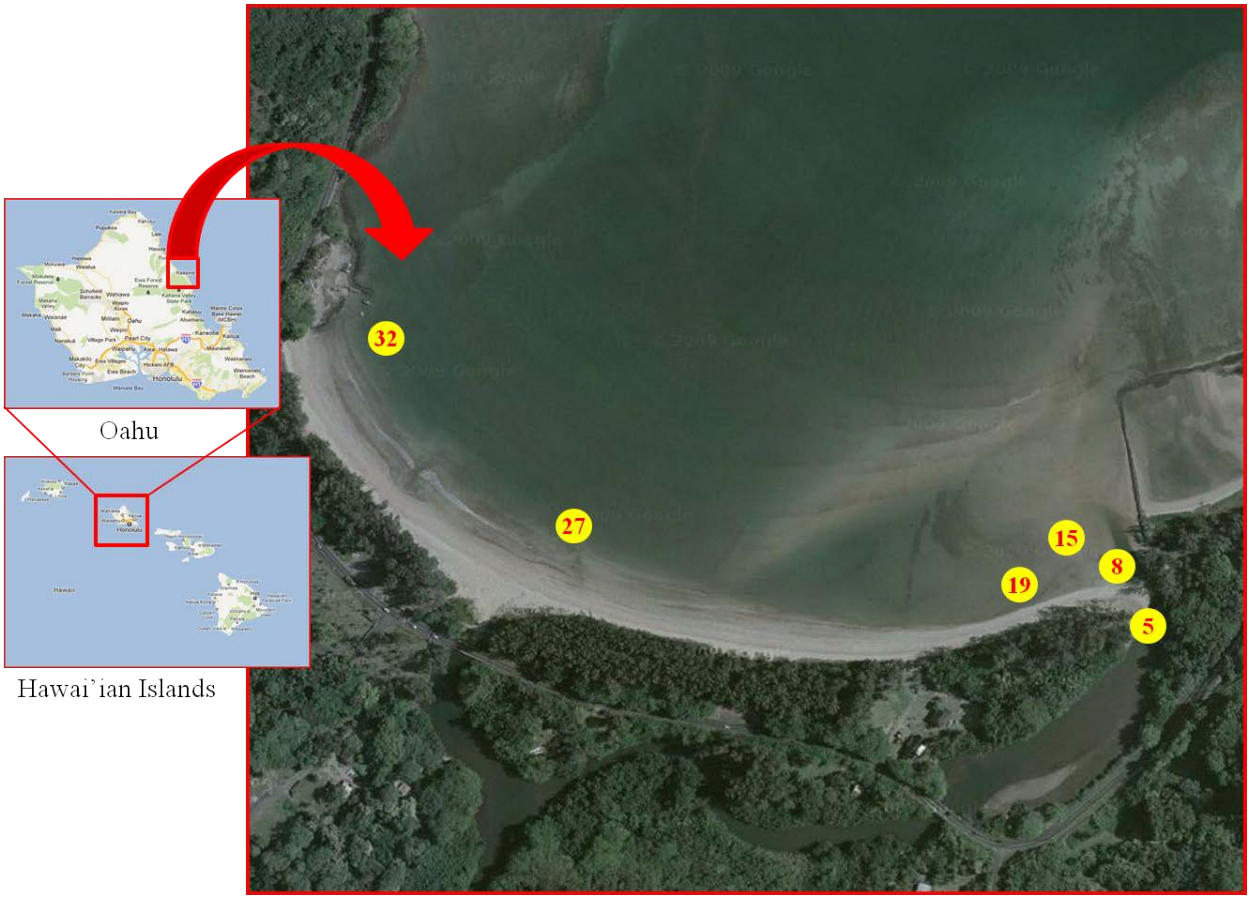

Figure 4. Sampling stations for a salinity transect (20 July 2010) from the Kahana Bay, Oahu, HI, USA. Station designations refer to their salinity (PSU).

DOC Analyses DOC was quantified by wet chemical oxidation on 2 mL sample volumes, using concentrated and cleaned sodium persulfate (Osburn and St-Jean 2007). Limit of detection via this method is 12 µmol L^{-1} C and reproducibility was <5%. Potassium hydrogen phthalate was used as a calibration standard for DOC concentrations over a range of 83 to 1,666 µM.

Lignin Analyses Presence and degradation state of terrestrially-derived OM (*i.e.* lignocelluloses) was determined by measuring lignin concentration and determining its relative degree of oxidative degradation. Lignin was measured as its component acid, aldehyde, and ketone phenols after microwave assisted CuO-oxidation (Louchouarn et al. 2000, Goni and Montgomery 2000). Phenols were extracted into ethyl acetate, redissolved into pyridine, derivatized, and then analyzed by GC/MS on a Varian 431-220MS using a DB-5 ms column. Lignin phenols were quantified against a standard curve of each of eight phenols released during the oxidation procedure (vanillin, vanillic acid, acetovanillone, syringealdehyde, syringic acid, acetosyringone, p-courmaric acid, ferulic acid). As there is currently no accepted radiotracer

method for measuring bacterial lignin metabolism, ratios of vanillic acid to vanillin ($Ac:Al_v$) content were used to indicate oxidative degradation (Hedges and Mann 1979).

Absorption and Fluorescence Spectroscopy Relative aromatic character of refractory dissolved organic carbon (DOC) was measured by its absorptive and fluorescent properties. Spectral absorption (200-800 nm) was measured on a Varian spectrophotometer and excitation-emission matrix (EEM) fluorescence on a Varian Eclipse spectrofluorometer on 0.2 μm filtrates from water samples. EEM fluorescence was measured on filtrates for DOM and on 0.1 N NaOH extracts of 0.7 μm GF/F filters for particulate organic matter (POM) fluorescence (Osburn et al. in prep); the appropriate Varian Eclipse instrument corrections applied and fluorescence data were reported in Raman-normalized quinine sulfate equivalents (QSE) in ppb, corrected for sample absorption (*i.e*. inner filter effects). All EEM data were modeled using the Parallel Factor Analysis (PARAFAC) procedure to decompose the fluorescent matrices into fluorescent components (Stedmon and Markager 2005, Stedmon and Bro 2008). Optical data processing was performed using Matlab software.

HIX/BIX Indices The biological index or BIX (Huguet et al. 2009) was calculated using the ratio of emission intensities at 380 nm and 430 nm at an excitation of 310 nm. BIX values >1.0 correspond to freshly produced DOM of algal or microbial origin, whereas values <0.6 indicate little freshly produced material. DOM humification generally increases its aromaticity, hence a humification index, HIX, was developed (Zsolnay et al. 1999). HIX was calculated from the ratio of two integrated emission wavebands: 435–480 nm to 300–345 nm, at 255 nm. HIX values <10 correspond to relatively non-humified DOM (Birdwell and Engel 2009) and generally increase with degradation. HIX also shows a direct correlation with aromaticity and is inversely correlated with carbohydrate content (Kalbitz et al. 2003).

Demarcation of Salinity Fronts, Salt Wedges and Confluence Zones Change in salinity over relatively narrow spatial scales was used to identify an area as a front, wedge or zone of confluence. Vertically stratified salt wedges (*e.g.* MSY-1, MSY-3 in the Gulf of Mexico, Figure 1; 6A in Charleston Harbor, Figure 2) were sampled by lowering the CTD rosette (with sample collection bottles) across the interface while firing the bottle closing mechanisms sequentially. The horizontally stratified front at the confluence between Ashley and Cooper Rivers in Charleston Harbor (Front, Figure 2) was sampled by towing the CTD rosette across the interface while firing the sampling bottles (1 m below sea surface). Collection bottles that contain water samples whose salinity was intermediate between water mass end members were considered representative of frontal samples. The transitional interface between fresh/brackish water and marine water masses was determined from previous sampling at Kahana Bay and was sampled from shore and by wading onto a shoal extending out from the Kahana River mouth. Salinity was measured with a refractometer and that which is intermediate between the river mouth (*e.g.* 5 PSU) and the nearshore water mass (25 PSU) was considered frontal boundary or transitional water samples. During previous sampling events (Montgomery et al. 2011), we found this mixing typically occurs over a shallow (0.5-1 m deep) shoal (inset in Figure 3) where stream flow and wave action mix the water masses over relatively small spatial scales (meters).

Mixing Experiments Small scale mixing experiments were performed for bacterial production by mixing different water masses (0.5 mL each of two water masses) of station MSY-3 at the

Gulf of Mexico, including 5/8, 5/22, 8/22, 22/29, 29/36, and 5/36 PSU. Substrate mineralization rates were only measured using the 5/29 PSU mixture for comparison with that for each individual salinity. A large scale mixing experiment (20 L) was performed using the end members (surface and bottom water) of the vertical profile across the salt wedge at station 6A (13.9 and 28.5 PSU, respectively) in the Cooper River of Charleston Harbor. Samples were incubated in 4 L carboys (ambient temperature) and periodically sampled (*e.g.* 0, 5, 15.5, 19.5, 22.5 h) for bacterial production. Mineralization rates were only determined for the T_0 mix and end members because the assay incubation time was 48 h. For the Kahana Bay sampling, Kahana River water (2 PSU) was mixed with Pacific Ocean water (35 PSU), incubated in the laboratory (20 L, University of Hawaii), and then sampled at T_0 and T_f (48 h) for bacterial production. Mineralization rates were only determined for T_0 mix and end members.

Data Interpretation We expected that if there is no effect of water mass interface on TNT metabolism, then the relationship between salinity and TNT metabolism (i.e. mineralization) would be conservative. That is, it would not be different from a linear mixing of freshwater and marine end members with respect to salinity. When there was enhanced or inhibited metabolism different from that which was expected from straight mixing, this was seen on a graph comparing these parameters with salinity. Those biogeochemical parameters that could be measured on similar spatial and temporal scales (*e.g.* DOC fluorescence, bacterial production etc.) and that demonstrated a similar pattern of change with respect to salinity were considered candidates for further study, as they were putative factors that control TNT metabolism in nature.

RESULTS AND DISCUSSION

This limited scope project primarily focused on demonstrating that frontal boundaries have enhanced rates of TNT mineralization, bacterial metabolism (*i.e.* heterotrophic production) and degradation of refractory OM (*e.g.* TNT, PAH, lignin). In addition, study of biogeochemical features of the interface that control these microbial rates and what cellular changes in the microbial assemblage occur in these environments (*e.g.* organotolerance) was initiated. If TNT metabolism rate can be related to heterotrophic production or OM transformation, then knowledge of general production and cycling rates may be applied to modeling TNT attenuation. Three coastal water surveys and mixing experiments between water mass end members were performed to address this topic.

Bacterial Production

Gulf of Mexico

Water samples were collected (via CTD rosette) from a depth profile across a salt wedge just outside the Mississippi River mouth in the Gulf of Mexico (MSY-3). There was a thin brackish water lens (0.5 m below surface) above an interfacial water layer ca. 1.5 m thick where salinity increased from 5 to 27 PSU (Figure 5). The bottom water mass (to ca. 7 m depth) was full strength seawater (ca. 36 PSU). Heterotrophic bacterial production decreased dramatically from 14.7 (+/- 6.9) μg C L^{-1} d^{-1} at the surface (0.4 m below Air-Sea Interface (ASI)) to 3.31 (+/- 0.51) μg C L^{-1} d^{-1} at 1 m below ASI before increasing back to 8.67 (+/-0.41) μg C L^{-1} d^{-1} in the bottom water (7.1 m depth; Figure 6). Though the water mass at this interface 1 m below the ASI had

the lowest bacterial production, this assemblage was also the least inhibited by naphthalene addition (*i.e*. most organotolerant) (Figure 6). It may be that the osmotic stress of existence at the interface resulted in lower overall productivity by the natural assemblage but that the assemblage that was able to handle such stress was resistant to further chemical stress of the naphthalene addition (organotolerant).

An additional mixing experiment was performed by combining various water mass samples (1:1 mix) from different salinities in the depth profile of station MSY-3. Bacterial production of the new mixture was then compared with the average of the two sources masses. In general, when the difference in salinity between the two water masses was larger (*e.g.* 8 verses 22; 5 verses 22; 5 verses 36 PSU), bacterial production was more inhibited (Figure 7) relative to an average of the two source masses (expected = 100%). When the salinity difference between the two masses was smaller (*e.g.* 29 verses 36; 5 verses 8 PSU), measured bacterial production was similar to what would be expected (near 100%). However, when the 22 PSU sample from the interface (1 m below the surface) was mixed with the 29 PSU sample (1.8 m below surface), bacterial production was actually stimulated (126% +/-4). *This observation supports the hypothesis that one water mass may provide growth limiting nutrients or factors for an adjacent water mass.* This nutrient effect may be trumped by decrease in overall production at higher osmotic stresses where there is greater difference in salinity between water masses.

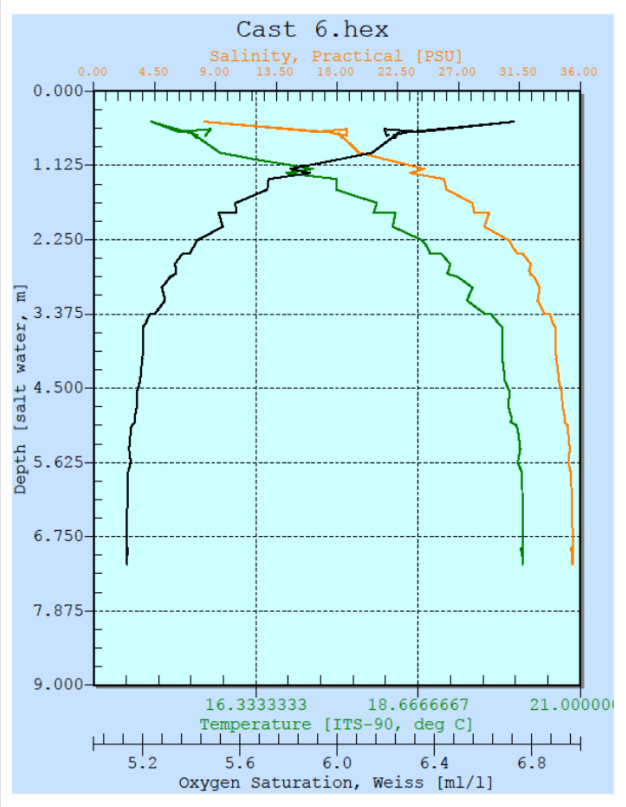

Figure 5. CTD cast depth profile (m) of the water column at station MSY-3 in the Gulf of Mexico just outside the mouth of the Mississippi River (16 March 2011) including salinity (PSU), temperature (^{o}C) and dissolved oxygen concentration (mL L^{-1}).

Charleston Harbor

Water samples were collected by towing a CTD rosette (1 m below ASI, Figure 8A) and sequentially firing the collection bottles across a tidal front at the confluence of Ashley and Cooper River in Charleston Harbor (during ebb tide). The front was visible on the surface and appeared to be a few meters wide (Figure 8B). Though the spatial distance (several meters) and salinity difference between the Ashley River (26.4 PSU) and Cooper River (24.2 PSU) was relatively narrow (Figure 9), there was almost a two-fold difference in bacterial production across this interface (18.9 +/- 2.18 verses 9.76 +/- 1.09 µg C L^{-1} d^{-1} comparing bottles 1 and 4; red triangles, Figure 10). *This demonstrates that there are large differences in metabolic capacity of the bacterial assemblage (and thus contaminant degrading potential) across these relatively small spatial areas within coastal estuaries.*

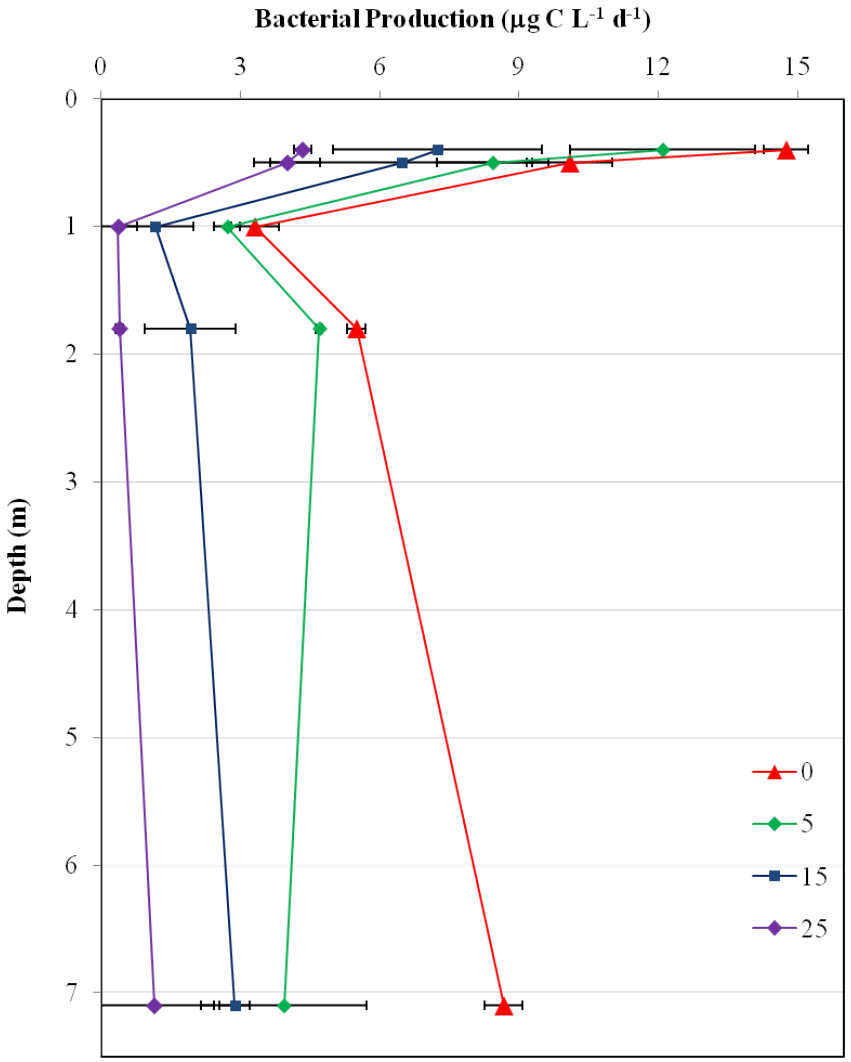

Figure 6. Bacterial production (µg C L^{-1} d^{-1}) with depth (m) at station MSY-3 in the Gulf of Mexico with various amount of naphthalene added (0, 5, 15, and 25 µg mL^{-1}) to determine relative organotolerance.

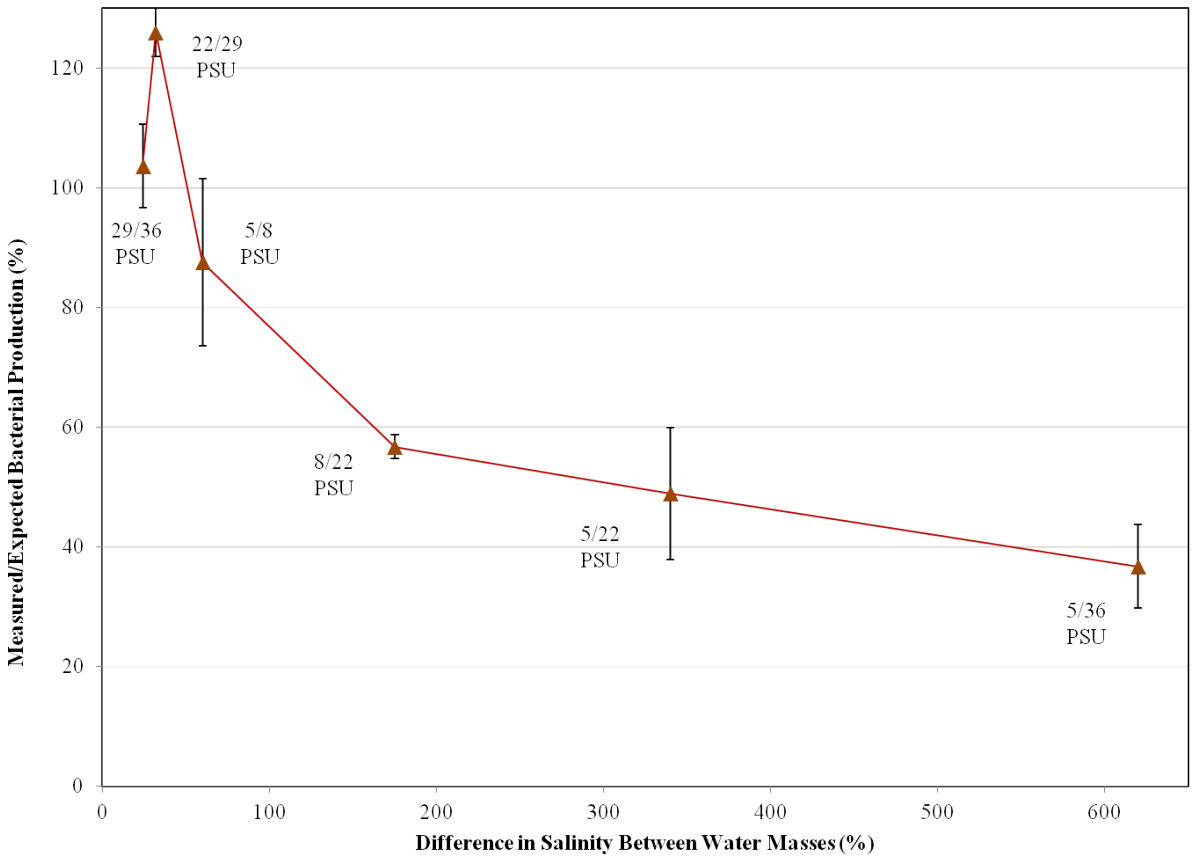

Figure 7. Bacterial production measured in water mass mixtures relative to the expected average of the source water masses (% of expected production) relative to percent difference in salinity between water masses at MSY-3 in the Gulf of Mexico, USA.

Figure 8. A CTD rosette with sampling bottles (A) was towed across the front between the Cooper and Ashley river (B) to sample this interface (26 June 2011) in Charleston Harbor, SC, USA.

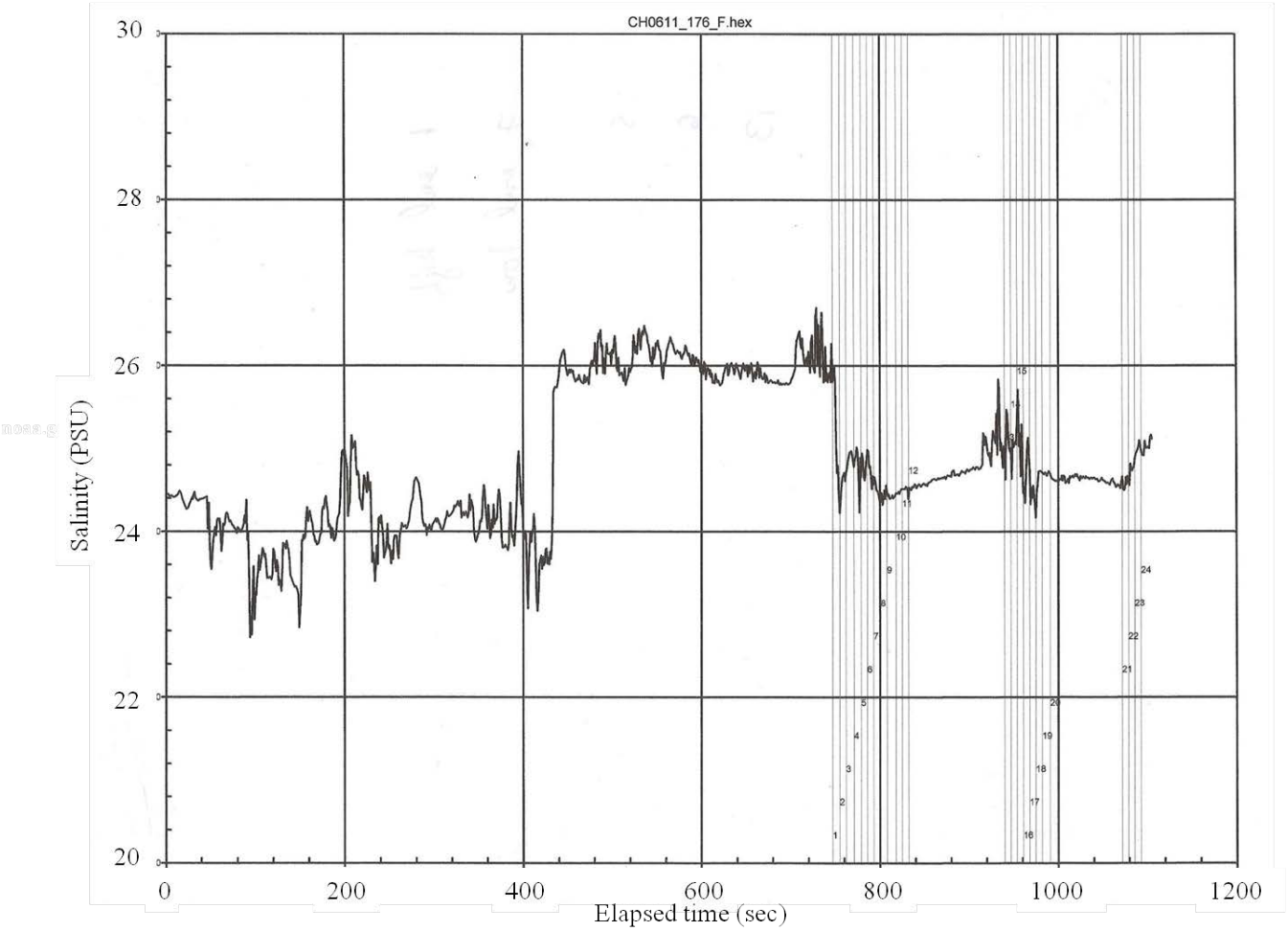

Figure 9. Salinity (PSU) over time (sec) as measured by the CTD rosette towed through the front between the Cooper and Ashley Rivers (1 m below sea surface; 26 June 2011) in the Charleston Harbor, SC, USA. Numbered vertical lines represent the firing of individual sampling bottles (1-24).

Kahana Bay

Surface water was collected by hand from the Kahana River, across a mixing area at the mouth of the river, along a subtidal area of the Kahana Bay to the marine end member of the Pacific Ocean (1 August 2011). Bacterial production in water was highest in the Kahana River (72.8 +/- 11.6 μg C L^{-1} d^{-1}, 2 PSU) and lowest at higher salinity (24.1-29.0 μg C L^{-1} d^{-1}, 13-35 PSU; Figure 11). Naphthalene addition (5 μg mL^{-1}) inhibited bacterial production the most at the marine station (37 +/- 14%; 35 PSU) and the least at the river mouth (13 +/- 8%, 5 PSU; Figure 12). Production was actually stimulated by naphthalene in the Kahana River sample by 26 (+/- 18) % (2 PSU; Figure 12). In a mixing experiment involving the end member water masses (i.e. 2 and 35 PSU), bacterial production was initially (T_0) intermediate between the two end members though 38% below their average (Table 1). *However, after 48 h (T_f), production of the mixture was actually 62% higher than the average of the two end members suggesting that*

growth stimulation of the natural assemblage has a time component or may require some period of adaptation.

Degradation of Dissolved Organic Carbon

Kahana Bay

Dissolved organic carbon (DOC) concentration was measured in surface water and compared with bacterial production along the Kahana Bay transect. The line connecting the two end members (2, 35 PSU) represents the expected DOC concentrations at intermediate salinities if the relationship is the result of conservative mixing, that is, DOC is produced at or before the riverine station and then is diluted out by low DOC seawater. However, intermediate salinity concentrations are all lower than would be expected (especially by 5 PSU; Figure 13) suggesting that there is some other removal mechanism for DOC in this ecosystem. *Bacterial production correlates relatively well (R^2 = 0.79) with DOC concentration along the transect suggesting that it may support heterotrophic bacterial growth, and thus DOC appears to be removed from the ecosystem by microbial metabolism* (Figure 14). Understanding the general relationships between DOC metabolism, bacterial production, and TNT metabolism, may allow use of the larger body of knowledge of DOC metabolism to predict the fate of contaminants in that same type of ecosystem.

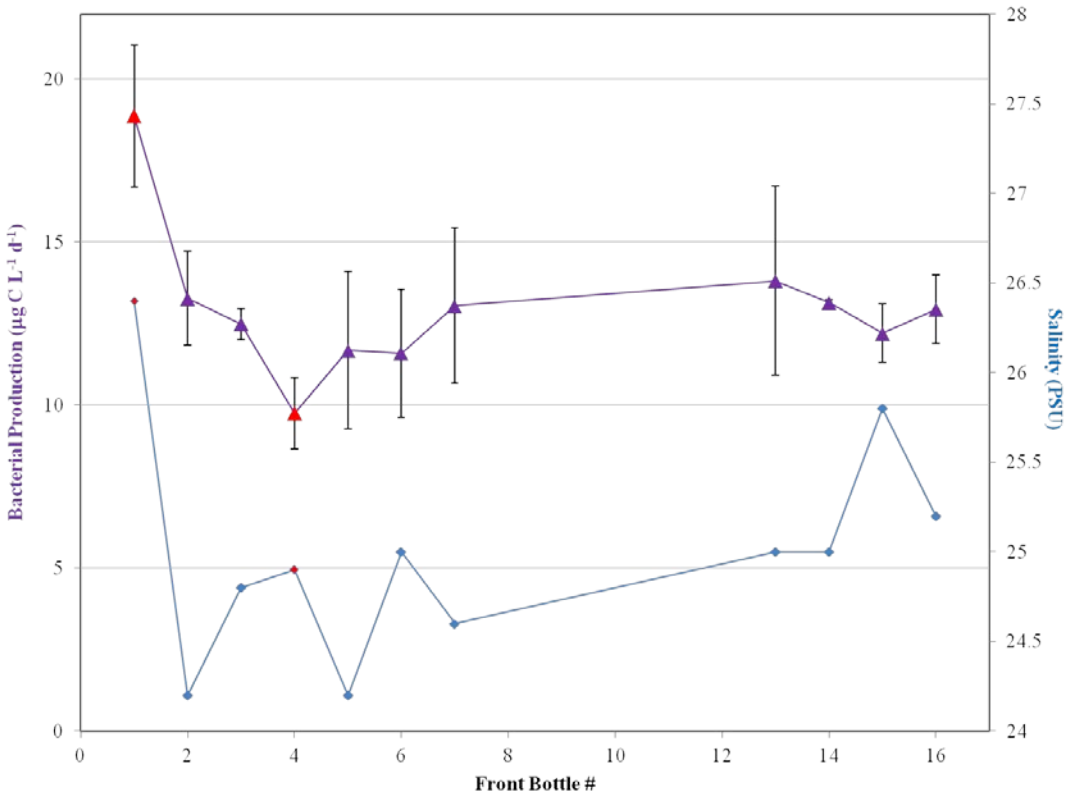

Figure 10. Bacterial production (µg C L^{-1} d^{-1}, left axis) and salinity (PSU, right axis) of water sampling bottles collected along the front between the Cooper and Ashley River (26 June 2011) in Charleston Harbor, SC, USA.

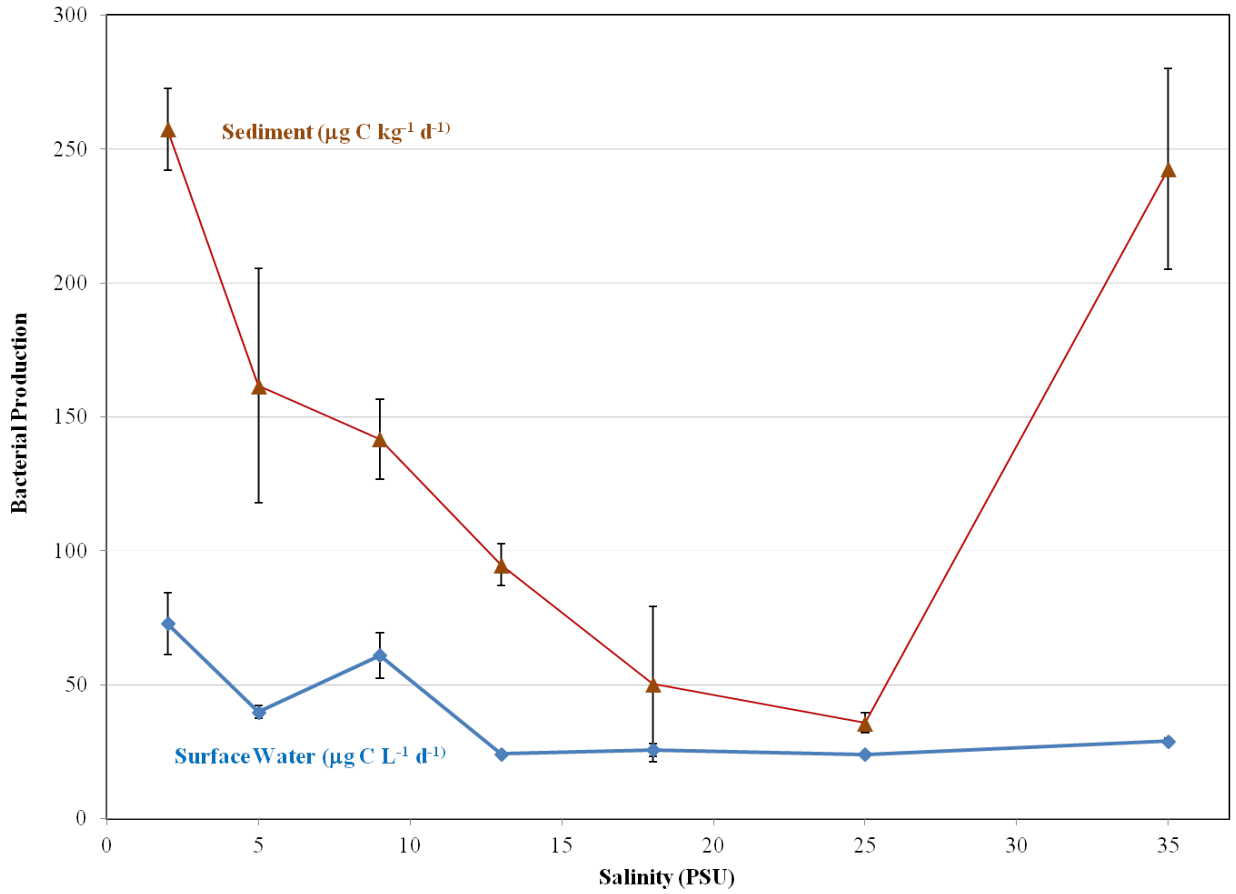

Figure 11. Bacterial production for surface water (blue line, µg C L^{-1} d^{-1}) and sediment (brown line, µg C kg^{-1} d^{-1}) collected along a salinity transect of Kahana River and Bay, Oahu, HI, USA (1 August 2011).

Table 1. Bacterial production (µg C L^{-1} d^{-1}) in surface water from Kahana Bay, Oahu, HI, USA (1 August 2011) for salinity transect end members (2, 35 PSU) and a 1:1 mix of these two samples (2/35 mix) at the start of incubation (T_0) and after 48 hours (T_f).

	Bacterial Production (µg C L^{-1} d^{-1})					
Time	2 PSU		2 /35 mix		35 PSU	
	AVG	SD	AVG	SD	AVG	SD
T_0	73	12	31	5.6	29	1.0
T_f	16	2.4	21	1.2	10	0.9

	% Stimulated above mix AVG
T_0	-38%
T_f	+62%

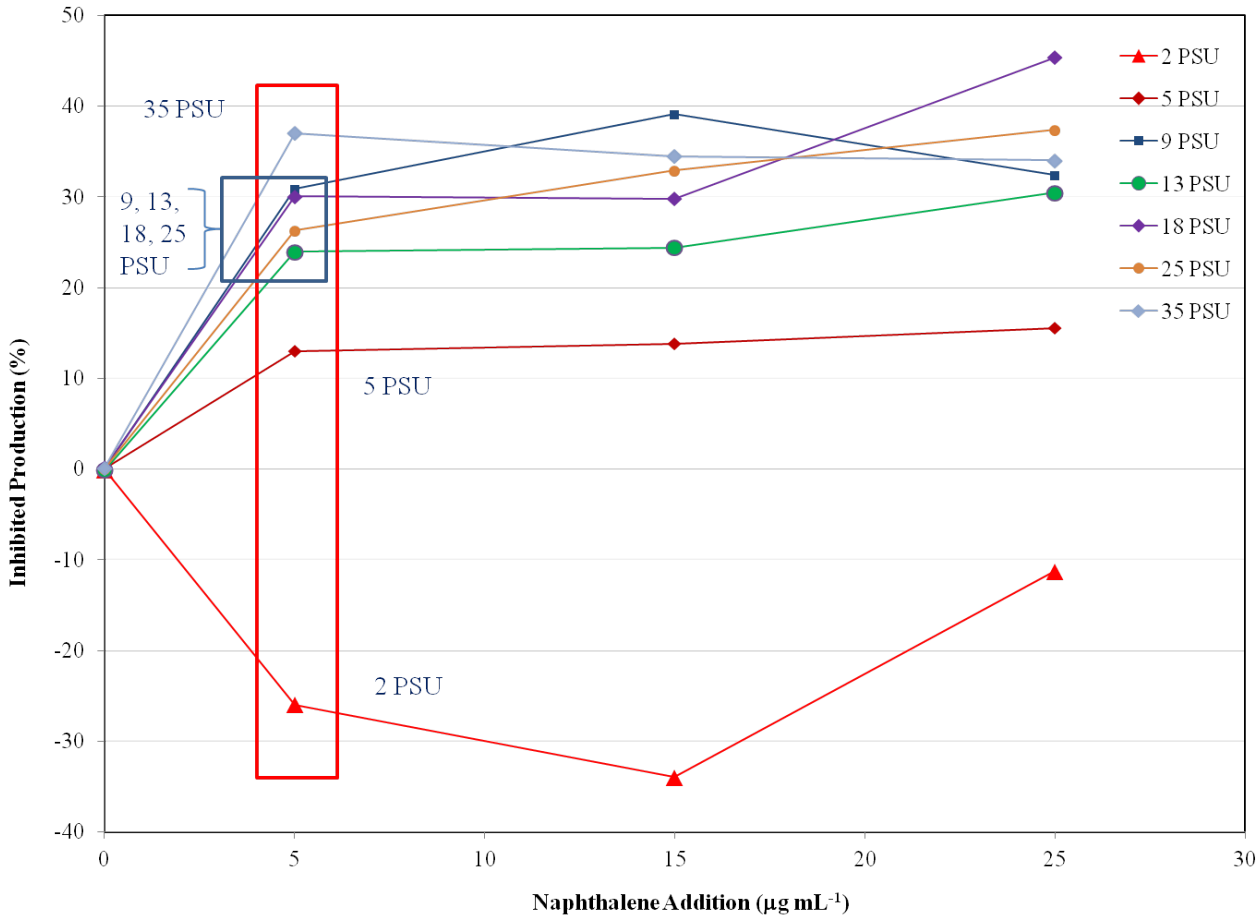

Figure 12. Effect of naphthalene addition (µg mL^{-1}) on bacterial production (% inhibition) of surface water from a salinity transect of Kahana Bay, Oahu, HI, USA (1 August 2011). The 5 µg addition is emphasized (red rectangle) as the effect may have been saturating above this concentration and is similar amongst estuarine stations (blue rectangle).

Degradation of Refractory Organic Matter (Aromatics)

Gulf of Mexico

Microbial mineralization of aromatic OM to CO_2 was measured using ^{14}C-radiolabeled TNT, naphthalene, and phenanthrene and compared with rates of bacterial production (Table 2). [Note the absolute value of the ratio is multiplied by 10^3 to make it a whole number for graphing purposes.] Highest ratio of mineralization (sum of all three substrates) to production was found in the 22 PSU interface sample in the MSY-3 depth profile (54, Table 2). Coincidentally, this 22 PSU sample also showed stimulation of bacterial production when mixed with the adjacent 29 PSU water mass (Figure 7). *This evidence supports the hypothesis that there may be increased aromatic metabolism by bacterial assemblages at interfaces between water masses.*

Table 2. Water, nepheloid (blue values) and sediment (brown values) were collected at various depths (m) in the Mississippi River and Gulf of Mexico (16 March 2011) and measured for salinity (PSU), temperature (^{o}C), dissolved oxygen concentration (DO, mL L^{-1}), bacterial production and mineralization rate of TNT, naphthalene (NAH) and phenanthrene (PHE) (water and nepheloid, µg C L^{-1} d^{-1}; sediment, µg C kg^{-1} d^{-1}), as well as, ratio of mineralization to production (x 10^{3}) for the individual and the sum of three carbon substrates. -- = Not Determined.

Station	Depth (m)	Salinity (PSU)	Temp (^{o}C)	DO (mL L^{-1})	Bacterial Production (µg C L^{-1} d^{-1}) or (µg C kg^{-1} d^{-1})		Mineralization (µg C L^{-1} d^{-1}) or (µg C kg^{-1} d^{-1})						Mineralization/ Production (x 10^{3})			
							TNT		NAH		PHE					
					AVG	SD	AVG	SD	AVG	SD	AVG	SD	TNT	NAH	PHE	SUM
MSY-1	0.2	3	15.7	6.8	3.29	0.03	0		--		--		0	--	--	--
	2.8	10	18.7	5.5	1.93	0.10	0		0.030	0.001	0.075	0.055	0	15.5	38.9	35.3
	8	36	20	5.1	2.22	0.01	0		--		--		0	--	--	--
	nepheloid	36	20	5.1	5.61	0.38	0		--		--		0	--	--	--
	sediment	36	--	--	3.12	1.27	0.139	0.021	--		--		45.5	--	--	--
MSY-2	0.2	0	9.6	7.9	15.4	3.77	0		0.050	0.007	0.152	0.036	0	3.3	9.9	13.1
	sediment	0	--	--	76.2	5.20	0.462	0.050					6.1			
MSY-3	0.4	5	14.7	6.9	14.7	0.48	0		0.038	0.009	0.122	0.030	0	2.6	8.3	10.9
	0.5	8	14.9	6.7	10.1	0.91	0.009	0.005	0		0.110	0.105	0.9	0	10.9	11.8
	1.0	22	16.6	5.9	3.31	0.51	0		0.012	0.003	0.167	0.067	0	3.6	50.5	54
	1.8	29	18.3	5.5	5.50	0.20	0		0.016	0.004	0.084	0.013	0	2.9	15.3	18.2
	7.1	36	20.2	5.1	8.67	0.41	0		0.018	0.010	0.282	0.061	0	2.1	32.5	34.6
	mix	5/29	--	--			0		0.018	0.006	0		0			--
	sediment	36	--	--	348	18	0.853	0.100	1.517	0.068	0.162	0.223	2.5	4.3	0.4	6.8

Charleston Harbor

In Charleston Harbor depth profiles, the highest ratio of mineralization to production for TNT was adjacent to the former Navy Yard (station 6A, Figure 15) at the salt wedge interface (3.2 m depth, 21 PSU; Table 3). For both naphthalene and phenanthrene, it was in the surface sample (0.9 m depth, 5.6 PSU) at the freshest water station (station 0, Table 3). When measuring TNT and phenanthrene mineralization across the tidal front, rates appeared to be most rapid at the beginning and end of each frontal boundary (Figure 16), that is, on both the Ashley River side and the Cooper River side of the mixing zone. These results may be more problematic to interpret because the narrow front structure is difficult to resolve with this type of sampling device (*e.g.* the CTD bottles themselves can become mixing vessels as they bring water from each water mass into the frontal interface). However, it was clear that rates of TNT and phenanthrene mineralization did increase as a function of soil humic acid content of DOM (Figure 17). Soil humic acids have greater aromaticity than other natural OM (Senesi and Sakellariadou 1994), thus environments that receive loads of aromatic natural OM may facilitate TNT and phenanthrene degradation. *Our results suggest there are large differences in aromatic mineralization rates across a relatively small, but heterogeneous sampling area (few meters).*

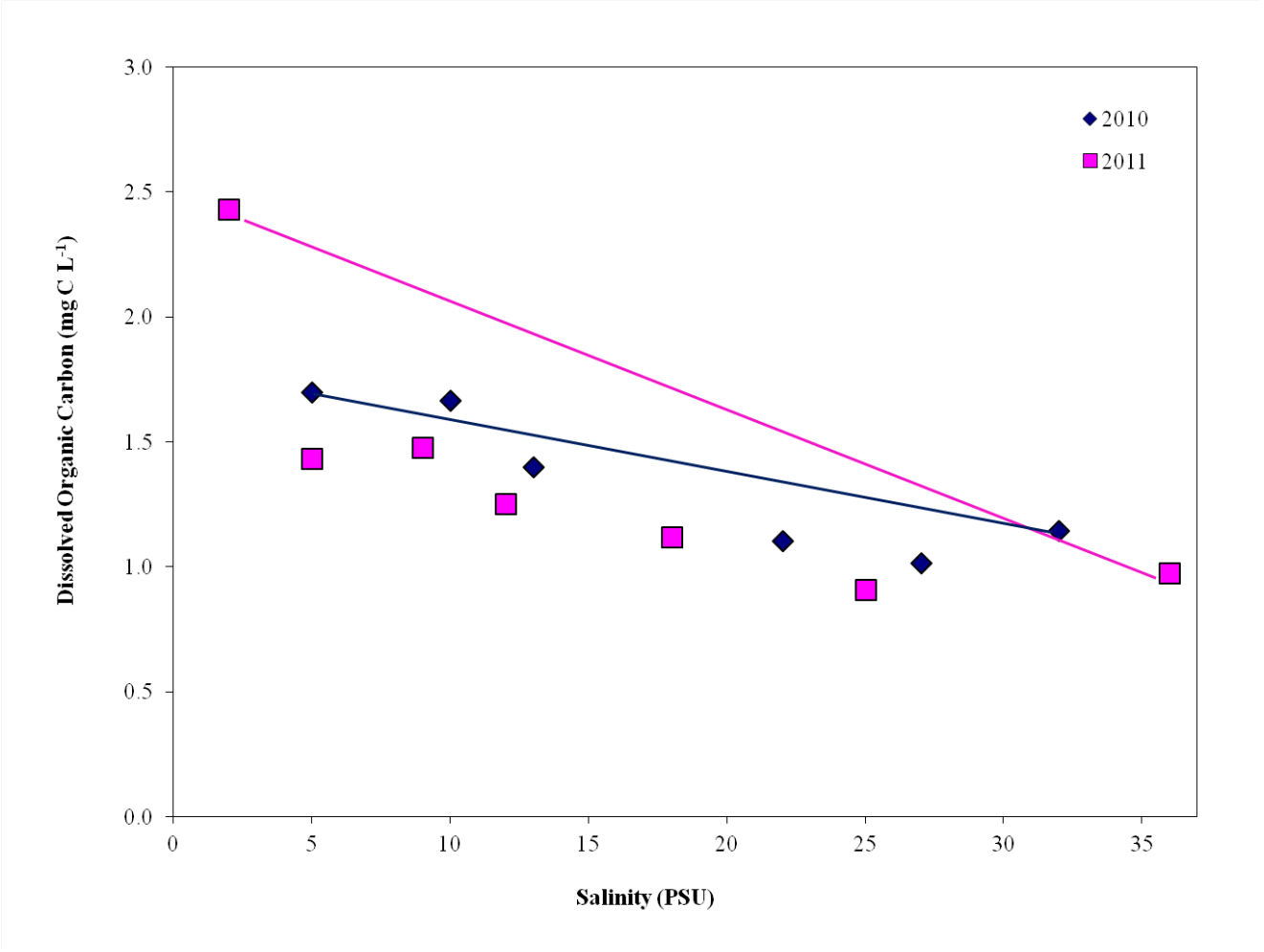

Figure 13. Dissolved organic carbon concentration (mg C L^{-1}) for surface water collected along salinity transects of Kahana River and Bay, Oahu, HI, USA (1 August 2011, red; 20 July 2010, blue).

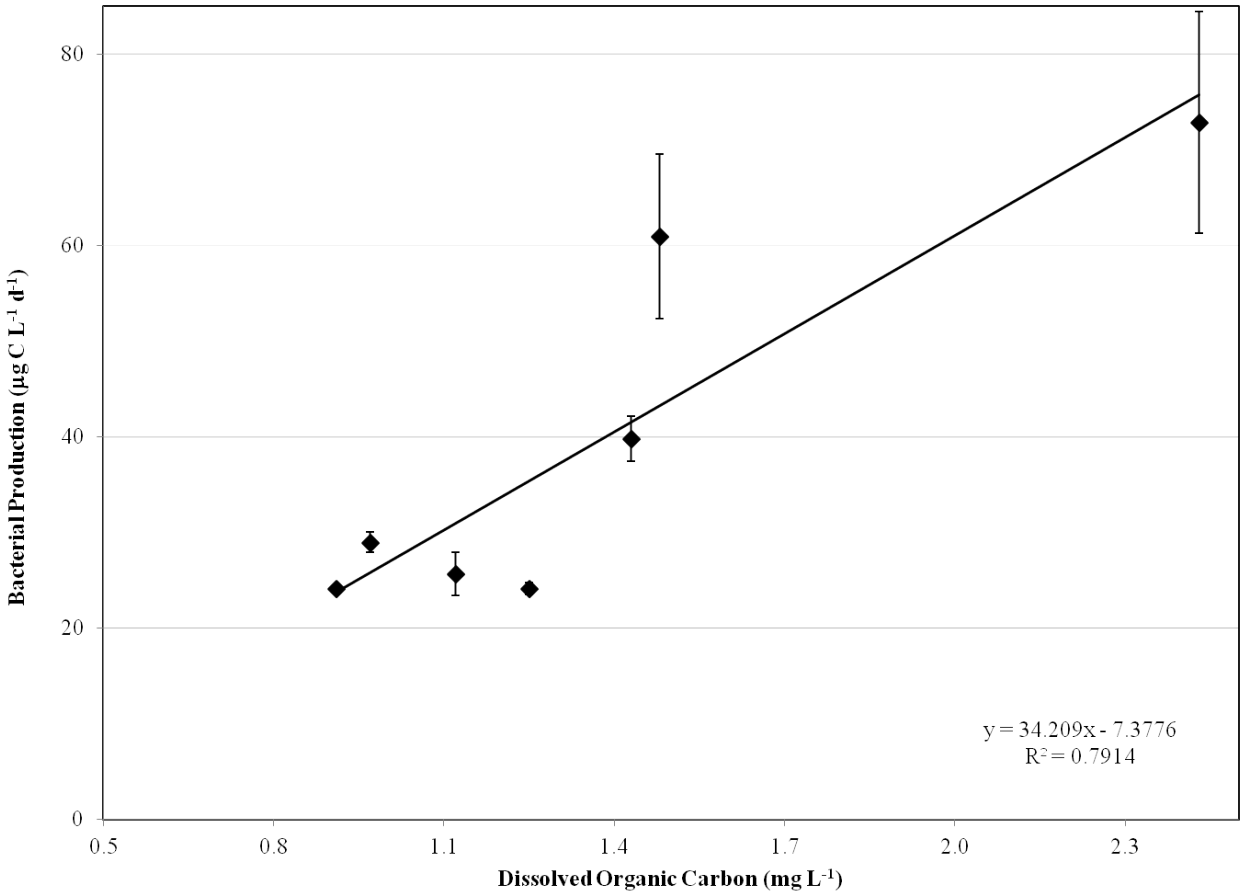

Figure 14. Bacterial production (µg C L^{-1} d^{-1}) correlated with DOC concentration (mg C L^{-1}) for surface water collected along a salinity transect of Kahana River and Bay, Oahu, HI, USA (1 August 2011).

Six components were derived from a Parallel Factor Analyses (PARAFAC) model of DOM and POM fluorescence for Charleston Harbor (C1-C6; Figure 18). C1 and C2 represented fresh and aged aquatic fulvic acids, respectively (Klapper et al. 2002) while C3 resembled amino sugars incubated in seawater (Biers et al. 2007), and C4 represented soil-derived humic acids (Fellman et al. 2010). C5 represented the amino acid tryptophan, while C6 represented the amino acid tyrosine. Fractional contribution of these components to overall fluorescence was compared to microbial mineralization rates. Surprisingly, the ratio of acid to aldehyde moieties for vanillyl phenols ($Ac:Al_v$), an index of oxidative degradation for lignin, was positively correlated with fraction of C1 in the DOM. Although no significant trends were found between TNT mineralization and DOM fluorescence, TNT mineralization rates did increase with the fraction of C1 of POM fluorescence ($r^2 = 0.61$, $p<0.05$) and decreased with fraction of C5 ($r^2 = 0.91$, $p<0.05$). *This strongly suggests that bacteria that are receiving inputs of aromatic natural OM low in nitrogen have an increased capacity to mineralize TNT*.

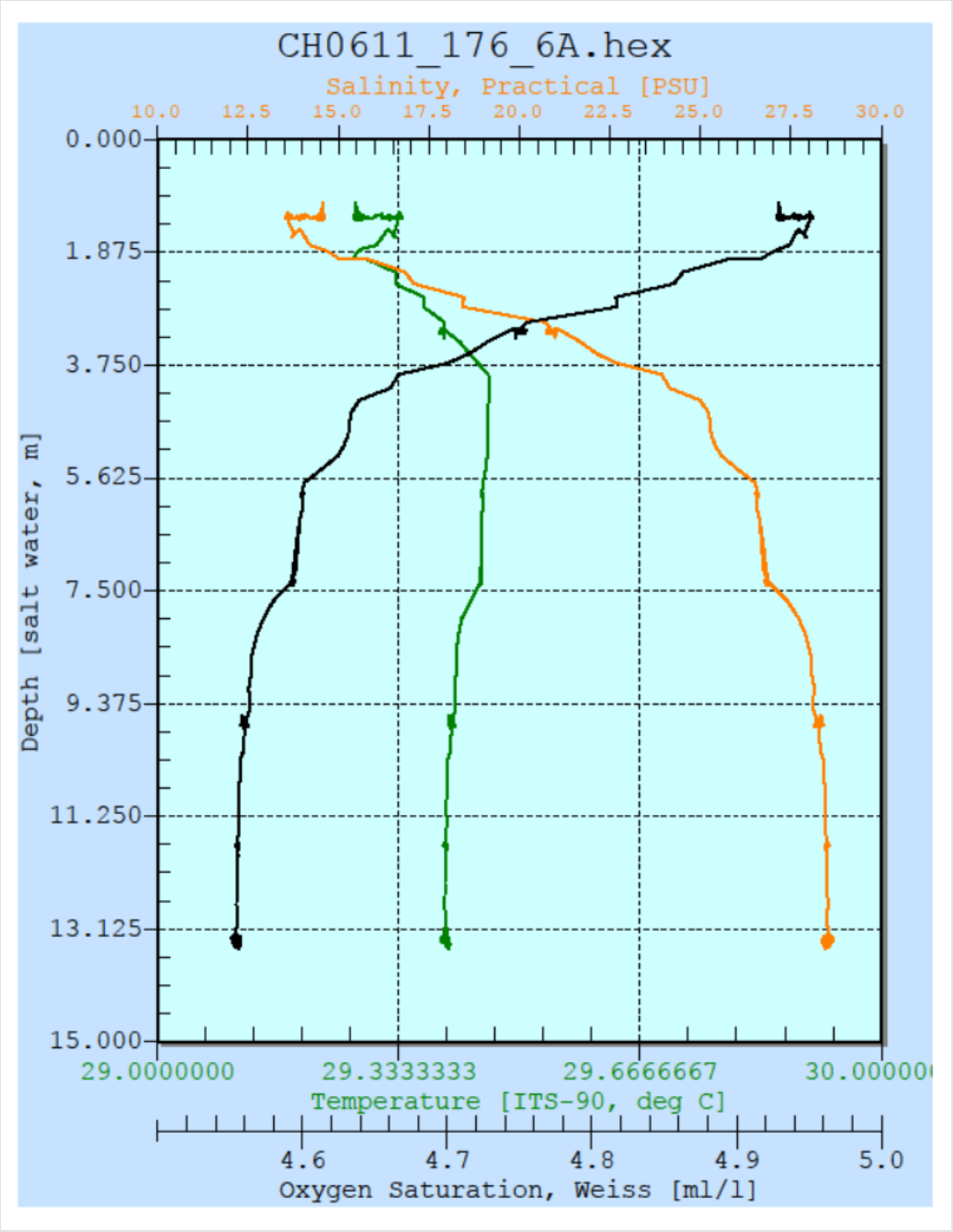

Figure 15. CTD cast depth profile (m) of the station 6A water column in the Cooper River of the Charleston Harbor system (26 June 2011) including salinity (PSU), temperature (^{o}C) and dissolved oxygen concentration (mL L^{-1}).

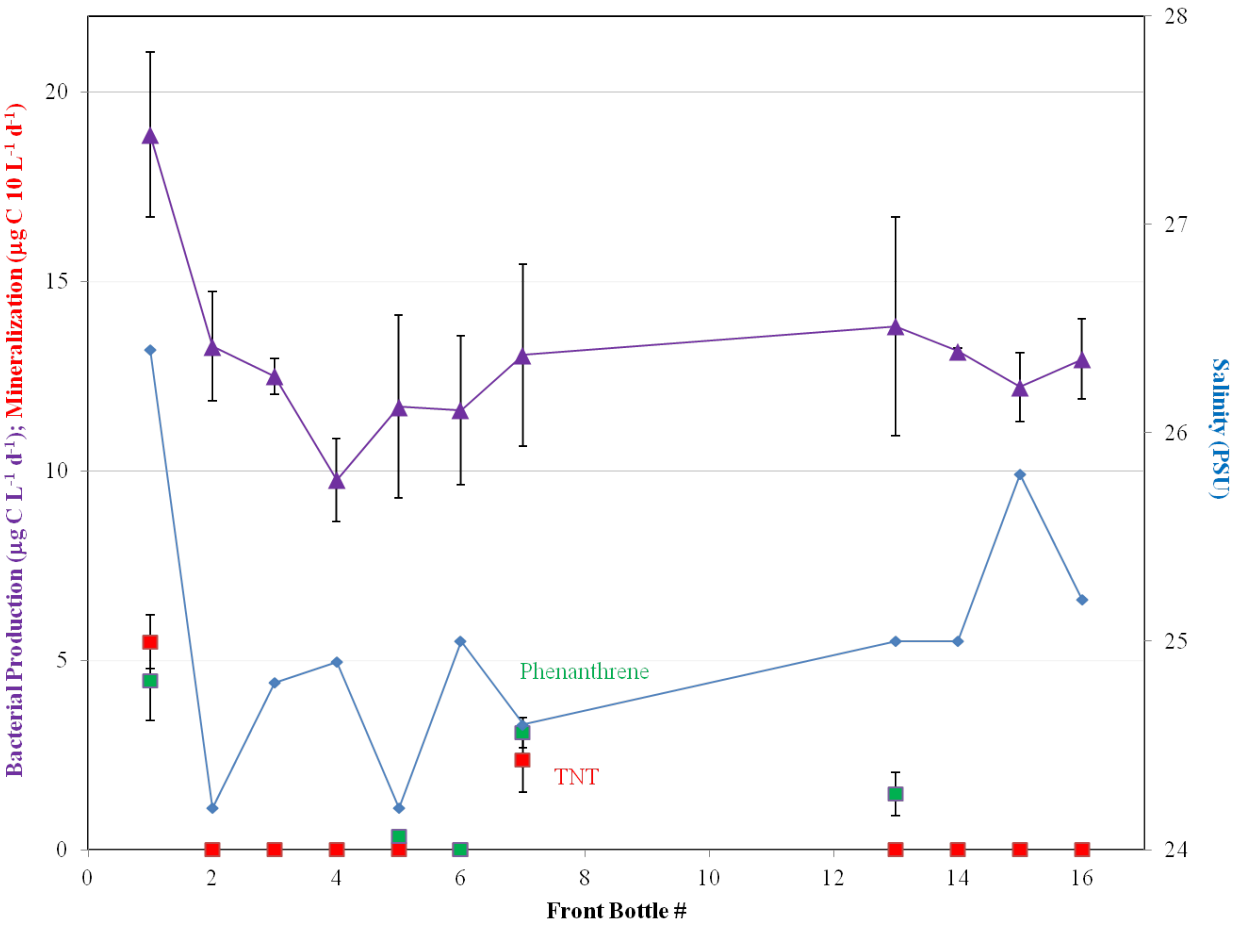

Figure 16. Bacterial production (µg C L^{-1} d^{-1}, purple), mineralization of TNT (red) and phenanthrene (green, µg C L^{-1} d^{-1}; left axis) and salinity (PSU, right axis) of sampling bottles collected along the front between the Cooper and Ashley Rivers (26 June 2011) in Charleston Harbor, SC, USA.

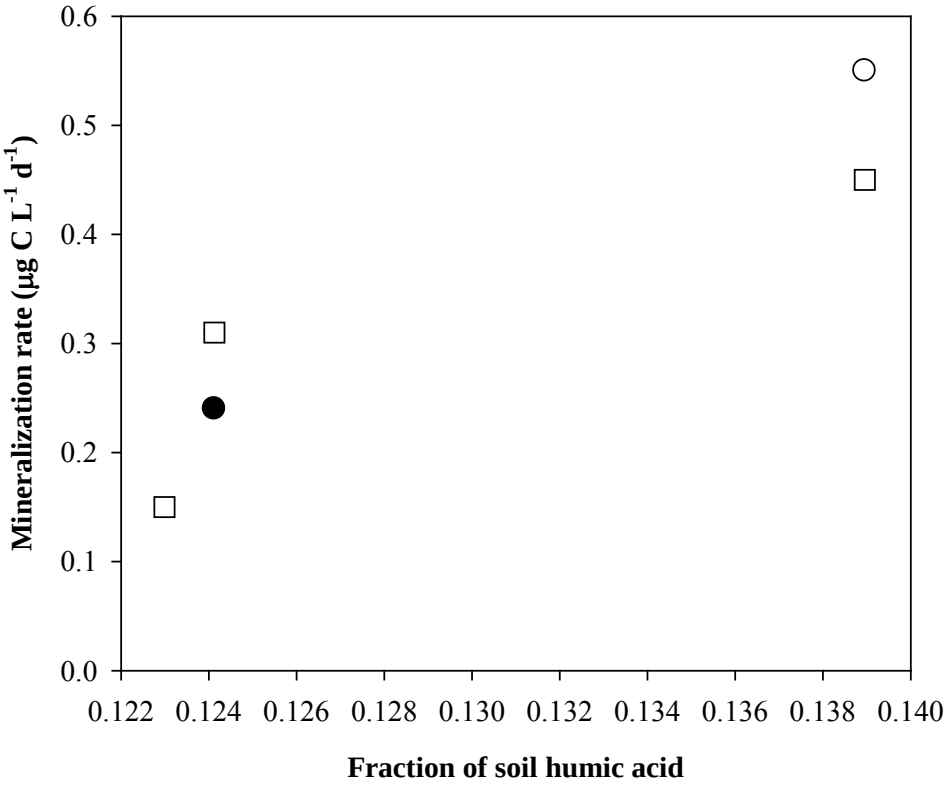

Figure 17. Fractions of soil humic acid of total DOM isolated from Kahana Bay water (1 August 2011) correlated with TNT (●) and phenanthrene (□) mineralization rates (µg C L^{-1} d^{-1}).

Kahana Bay

Unlike in Charleston Harbor and the Gulf of Mexico, TNT mineralization in both Kahana Bay water and sediment was often an order of magnitude higher than that for naphthalene or phenanthrene (Table 4). For surface water TNT mineralization, it is interesting to note that the highest mineralization rate of the survey was at the 13 PSU station (0.17 +/ 0.14 µg C L^{-1} d^{-1}) which was in the mixing zone of the estuary. This rate was very similar to that of the mixing experiment between the end members of 2 and 35 PSU (0.16 +/ 0.06 µg C L^{-1} d^{-1}) (Table 5). Likewise, the ratio of mineralization to production was also higher than would be predicted from averaging the two end members for both TNT and phenanthrene (Table 5). In addition, the highest sum of ratio of water mineralization to production (9.5, Table 4) was at station 9, which is also in the mixing zone near the river mouth. The second highest (7.2, Table 4) was seen with the sample that was a mix of the two end members. *These findings are further support of the hypothesis that there is enhanced aromatic degradation in mixing zones between water masses.*

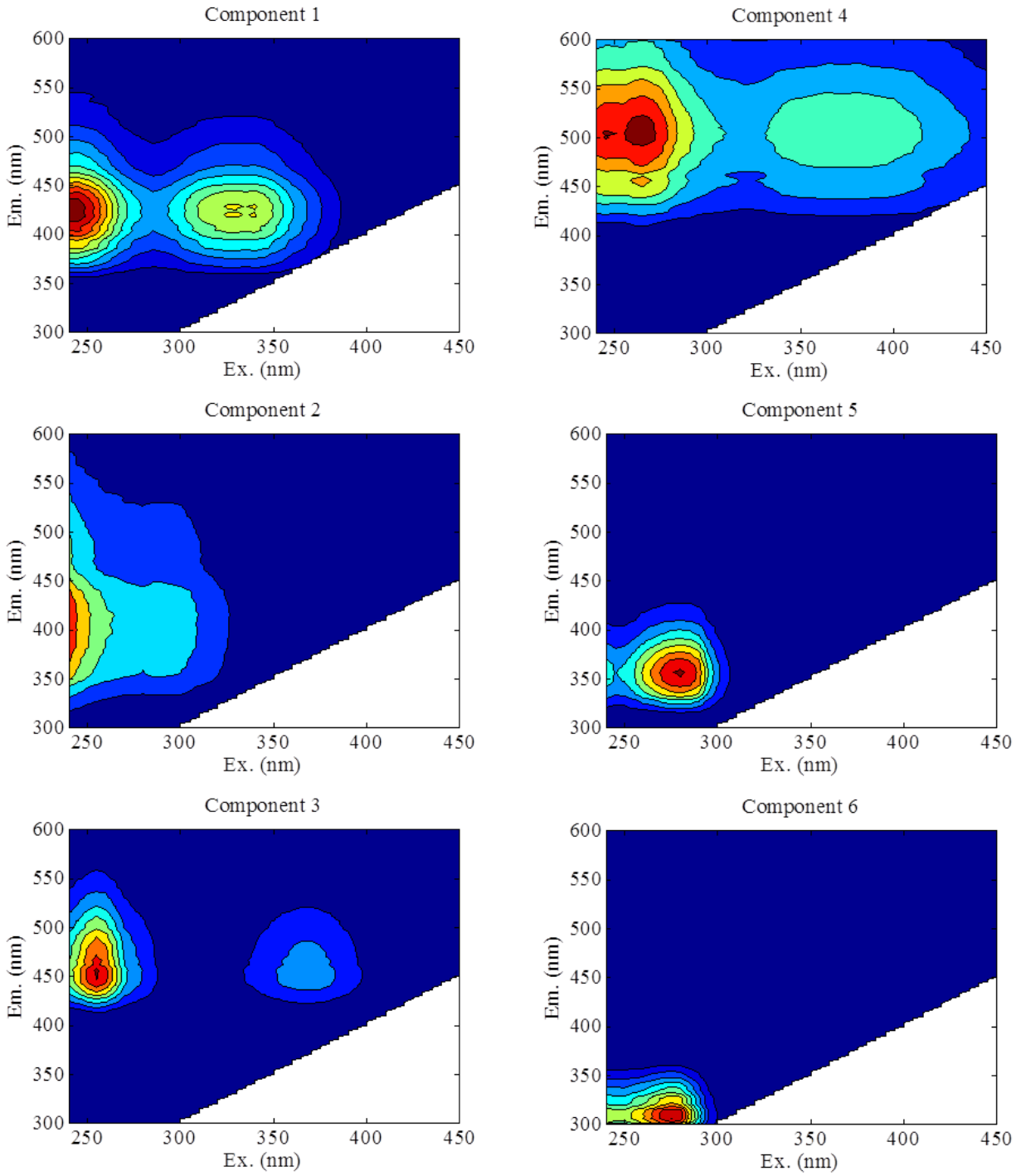

Figure 18. Six components derived from a PARAFAC model of DOM and POM fluorescence for Charleston Harbor, SC, USA: C1 and C2 represent fresh and aged aquatic fulvic acids, respectively; C3 resemble amino sugars incubated in seawater; C4 represent soil-derived humic acids; C5 represent the amino acid tryptophan; and, C6 represent the amino acid tyrosine.

Table 3. Water, nepheloid (blue values) and sediment (brown values) were collected at various depths (m) in the Charleston Harbor system (26 June 2011) and measured for salinity (PSU), temperature (°C), dissolved oxygen concentration (DO, mL L^{-1}), bacterial production and mineralization rate of TNT, naphthalene (NAH) and phenanthrene (PHE) (water and nepheloid, µg C L^{-1} d^{-1}; sediment, µg C kg^{-1} d^{-1}), as well as, ratio of mineralization to production (x 10^3) for the individual and sum of three carbon substrates. -- = Not Determined.

					Bacterial Production (µg C L^{-1} d^{-1}) or (µg C kg^{-1} d^{-1})		Mineralization (µg C L^{-1} d^{-1}) or (µg C kg^{-1} d^{-1})						Mineralization/ Production (x 10^3)			
							TNT		NAH		PHE					
Station	Depth	Salinity (PSU)	Temp (°C)	DO (mL L^{-1})	AVG	SD	AVG	SD	AVG	SD	AVG	SD	TNT	NAH	PHE	SUM
6A	1.3	13.9	29.3	4.94	10.3	1.93	0.26	0.21	0.23	0.03	0.11	0.03	25.2	22.3	10.7	70.9
	3.2	21	29.4	4.75	10.3	1.13	0.39	0.18	0.17	0.04	0.12	0.07	37.9	16.5	11.7	66.1
	13.3	28.5	29.4	4.55	12.5	0.67	--		0.09	0.02	--		--	7.2	--	--
	nepheloid	28.5	29.4	4.55	10.2	1.20	0.27	0.12	0.13	0.05	0		26.5	12.7	0	39.2
	sediment	28.5	29.4	--	43.1	3.41	--		1.03	0.27	0.14	0.03	--	23.9	3.2	--
0	0.9	5.6	30.5	5.07	6.79	0.45	--		1.09	0.29	0.53	0.20	--	161	78.1	--
	2.8	6.5	30.4	5.05	10.3	0.13	0		0.15	0.01	0		0	14.6	0	14.6
	5.5	10.4	29.9	4.99	11.0	2.28	0		--		0.08	0.03	0	--	7.3	--
6E	1	17.1	30.2	4.78	15.4	3.81	0.20	0.17	--		0.17	0.05	13	--	11	--
	2.6	23	29.5	4.69	10.4	1.03	0		--		0.21	0.04	0	--	20.2	--
	6.9	28	29.3	4.57	7.64	0.40	0.17	0.04	--		0.15	0.04	22.3	--	19.6	--
	nepheloid	28	29.3	4.57	6.96	1.67	0		0.05	0.02	0.10	0.02	0	7.2	14.6	21.8
	sediment	28	29.3	--	81.4	3.82	0		0.50	0.24	0.10	0.08	0	6.1	1.2	7.3
A3	1.3	23.3	29.3	4.7	2.61	0.30	0		--		--		0	--	--	--
	7.4	24.8	29.5	4.64	11.5	0.41	0		--		--		0	--	--	--
	12.4	28.8	29.3	4.56	--		0		--		--		0	--	--	--
	nepheloid	28.8	29.3	4.56	15.5	0.01	0		--		0.19	0.06	0	--	12.3	--

The humification index, HIX, is a DOM parameter that describes the "freshness" of recent plant biomass (HIX <5) or degree of humification (*e.g.* HIX 10-30 for soil DOM; Huguet et al. 2009). In the literature, HIX is positively correlated with aromaticity and, in Kahana Bay waters, shows a strong positive correlation with naphthalene mineralization (Figure 19 A). The biological index, BIX, also is a derived DOM qualitative parameter from fluorescence signals specific to microbially-derived humic substances, amino sugars, and proteins. Consequently, this parameter was negatively correlated with naphthalene mineralization in Kahana Bay (Figure 19 B). One explanation is that the presence of aromatic-poor algal and microbial carbon is not supportive of microbial propensity to degrade aromatic organic molecules, such as PAHs or TNT. Rather, natural assemblages that are exposed to aromatic OM (such as lignin) have this capacity.

Finally, in underlying sediment, TNT mineralization rate generally decreased from freshwater end member (2 PSU, 2.4 +/- 0.59 µg C kg^{-1} d^{-1}) to non-detect in the Pacific Ocean sample (35 PSU, Table 4) for the August 2011 sampling. During a previous sampling (20 July 2010), TNT mineralization rate in sediment was highest at mid salinity (18 PSU) and decreased towards each end member (Figure 20). Although both samplings showed patterns with salinity, the underlying control of these patterns may be less straightforward than in the water sampling as sediment is

vertically stratified with depth in addition to being influenced by the conditions of the overlying water column.

Table 4. Water (blue values) and sediment (brown values) were collected from Kahana River and Bay, Oahu, HI, USA (1 August 2011) and measured for bacterial production and mineralization rate of TNT, naphthalene (NAH) and phenanthrene (PHE) (water, µg C L^{-1} d^{-1}; sediment, µg C kg^{-1} d^{-1}), as well as, ratio of mineralization to production (x 10^3) for the individual and sum of three carbon substrates.

	Sediment Mineralization (µg C kg^{-1} d^{-1})						Mineralization/ Production (x 10^3)			
Station	TNT		NAH		PHE					
(PSU)	AVG	SD	AVG	SD	AVG	SD	TNT	NAH	PHE	SUM
2	2.4	0.59	0.16	0.028	0		9	0.6	0	1-
5	0.94	0.63	0.16	0.06	0.18	0.05	6	1	1	8
9	1.6	0.73	0.13	0.08	0		11	0.9	0	12
13	1.3	0.98	0.27	0.04	0		14	3	0	17
18	1.2	0.71	0		0		24	0	0	24
25	0.78	0.35	0		0		22	2	0	24
35	0		0.11	0.04	0		0	0.5	0	0.5

	Surface Water Mineralization (µg C L^{-1} d^{-1})						Mineralization/ Production (x 10^3)			
	TNT		NAH		PHE					
	AVG	SD	AVG	SD	AVG	SD	TNT	NAH	PHE	SUM
2	0.12	0.05	0.025	0.020	0.033	0.005	1.6	0.34	0.45	2.4
5	0		0.038	0.018	0.039	0.006	5.2	0.95	0.98	7.2
9	0.11	0.02	0.041	0.008	0.031	0.030	1.9	0.68	0.50	3.0
13	0.17	0.14	0.022	0.012	0.034	0.009	7.1	0.92	1.4	9.5
18	0.07	0.06	0.012	0.006	0		2.5	0.48	0	3.0
25	0		0		0.091	0.046	0	0	3.8	3.8
35	0.12	0.01	0		0.047	0.016	4.3	0.18	1.6	6.1

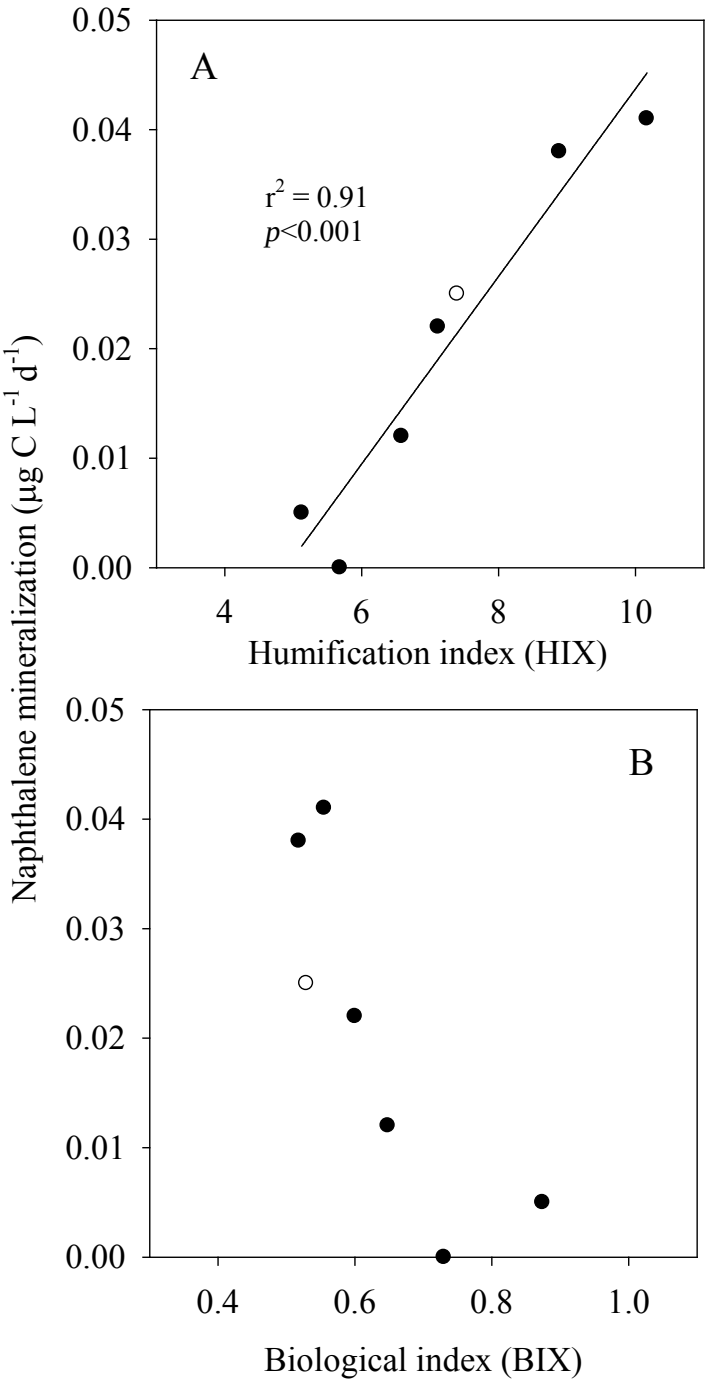

Figure 19. Organic matter (OM) collected from Kahana Bay water (1 August 2011) characterized using (A) HIX, the humification index, a derived parameter from DOM fluorescence that describes "freshness" of recent plant biomass (HIX <5) or degree of humification (*e.g.* HIX 10-30 for soil DOM); (B) BIX, the biological index, also is a DOM qualitative parameter derived from fluorescence signals specific to microbially-derived humic substances, amino sugars, and proteins. Both HIX and BIX indices compared with naphthalene mineralization rate (µg C L^{-1} d^{-1}) by the natural bacteria assemblage.

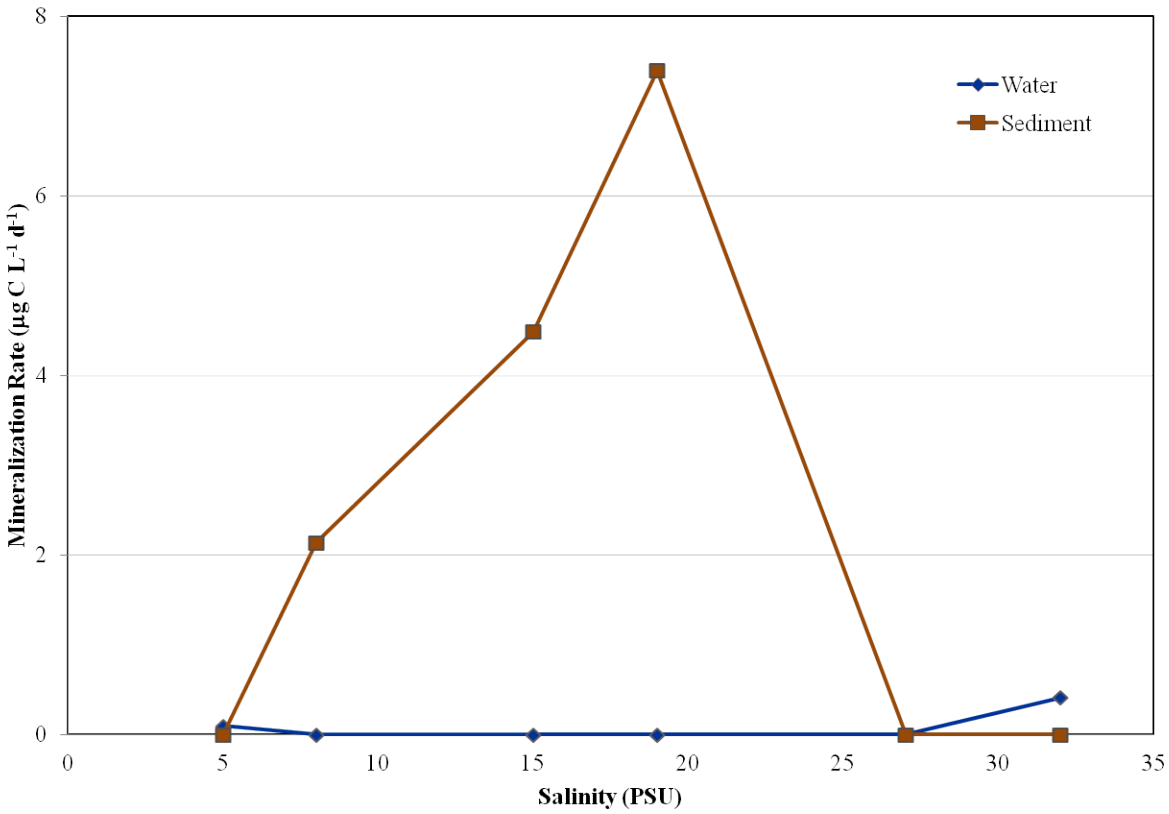

Figure 20. TNT mineralization rate for surface water (µg C L^{-1} d^{-1}) and sediment (µg C kg^{-1} d^{-1}) along a salinity transect (PSU) of Kahana River and Bay, Oahu, HI, USA (20 July 2010).

Table 5. Water (2, 18, 35 PSU) collected from Kahana River and Bay, Oahu, HI, USA (1 August 2011) was measured for mineralization rate of TNT, naphthalene (NAH) and phenanthrene (PHE, µg C L^{-1} d^{-1}) and then compared with that from a 1:1 mix of water from stations 2 and 35 PSU (2/35). TNT and phenanthrene mineralization and ratio of mineralization to production (x 10^3) were compared with that of individual end members (2, 35 PSU) and the estuarine station of similar salinity (18 PSU).

	Surface Water Mineralization (µg C L^{-1} d^{-1})					
	TNT		NAH		PHE	
Station	AVG	SD	AVG	SD	AVG	SD
2	0.12	0.05	0.025	0.020	0.033	0.005
2/35 mix	0.16	0.06	0		0.067	0.023
18	0.065	0.059	0.012	0.006	0	
35	0.12	0.01	0		0.047	0.016
	% Stimulated above mix AVG					
2/35	+33%		-50%		+68%	
	Mineralization/Production (x 10^3)					
	2	1.6	0.34		0.45	
$T_{0,f}$	2/35	5.2	0		2.2	
	35	4.1	0		1.6	

CONCLUSIONS AND IMPLICATIONS FOR FUTURE RESEARCH

According to the transition plan in our limited scope proposal, "*if the zones between water masses (e.g. frontal boundary, convergence, salt wedge) are found to be areas of the ecosystem with the most rapid microbial TNT incorporation and mineralization rates (proof of principle), the factors controlling TNT fate will be further examined in a follow-on core proposal*". We found evidence that water mass interfaces are sites of enhanced bacterial metabolism and TNT mineralization when examining salt wedges (Gulf of Mexico, Charleston Harbor), a frontal boundary (Charleston Harbor), and during mixing experiments between freshwater and marine end members (Gulf of Mexico, Charleston Harbor, Kahana Bay). Mineralization of aromatic contaminants also appears closely related with natural aromatic OM indices. In general, repeating these measurements seasonally and over different river flow regimes (at these sites and other Navy-relevant biomes) would make these important findings robust enough for publication in peer-reviewed scientific journals. Upon such validation, the applicability of such findings would be included to justify Monitored Natural Attenuation strategies or No Further Action decisions in cases where range emissions were exceeded by natural assemblage energetic metabolism. **Understanding the relationships between biogeochemical parameters and TNT biodegradation amongst various ecosystem (biome) types may allow the determination of attenuation rates even at DoD sites with limited access to water and sediment samples due to the presence of UXO.**

Specific issues involving follow-on research that we would address include criteria for site selection, technical sampling issues involving the original hypothesis, and further developing findings from the mixing experiments. Ideally, the aggregate of study sites would be both DoD-relevant and comprise and large cross section of coastal environments and biome types. Our collection of data could add to the body of knowledge of an ongoing or future site investigation, thereby reducing redundant chemical analyses and reference sampling cost to the DoD site manager. Seasonal sampling would address some of the natural variation in bacterial growth and mineralization rates in coastal ecosystems though this may be more important in temperate biomes than in tropical. Use of large research vessels makes it logistically easier to sample watersheds that include multiple UXO fields or active ranges (*e.g. R/V Cape Hatteras* for North Carolina, *R/V Sharp* for Chesapeake Bay). Selecting a wide variety of ecosystems for study may allow the application of our findings to similar site where we have not yet collected data. An example of balancing these considerations would be a 3-year plan (3-4 samplings per year) of seasonal samplings of two temperate biomes, *e.g.* coastal North Carolina (Cat Island/Pamlico Sound/Camp Lejuene) and Chesapeake Bay (Bloodworth Island/Tangier Island/Aberdeen), northern coniferous biome, *e.g.* Pacific Northwest (Ostrich Bay/Jackson Park), and fewer samplings of tropical biomes, *e.g.* Key West (POC: Dana Hayworth), Vieques (POC: Dan Waddill).

Technical sampling issues that would be addressed involve the spatial resolution of water column collection and determining the residence time of the mixed water mass. Relatively large heterogeneity (2-10 fold) was found with both bacterial production and TNT mineralization over small distances (m). Water collection bottles (20 L) on the shipboard CTD rosette were likely too large and vertically oriented to capture discreet water samples from the frontal interface (thus

acting as mixing chambers themselves). A smaller, horizontal sampling bottle may be more useful in future water collection at both vertical and horizontal fronts.

Finally, it may be useful to measure the residence time of a water mass at the frontal boundaries (possibly using natural abundance of uranium-thorium series nuclides). Although we may measure the salinity of a frontal water mass as being intermediate between the freshwater and marine end members (*e.g.* 18 PSU), we do not know when the end members were mixed (maybe days or weeks in well-stratified systems). During the mixing experiments between water mass end members, bacterial production was initially inhibited (T_0) but then recovered and was enhanced hours to days afterward (along with TNT mineralization). Incongruities between the results from mixing experiments (where the time since mixing is known) and those from similar salinity water masses collected at a frontal boundary in nature may be due to this issue. By addressing these technical and scientific issues and coupling these experiments to a sampling regime of DoD relevant ecosystems, we may get a large amount of information on many energetics sites in a relatively cost effective sampling and analysis strategy.

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LIST OF APPENDICES

Appendix A. TNT mineralization (as a rate, μg L^{-1} d^{-1}; % of added; and % d^{-1}) and incorporation efficiency (%) calculated from data provided in the literature for microbial assemblages in aqueous media (adapted from Montgomery et al. 2008). The associated rates of heterotrophic bacterial metabolism are rarely reported.

Appendix B. TNT mineralization (as a rate, μg kg^{-1} d^{-1}; % of added; and % d^{-1}) and incorporation efficiency (%) calculated from data provided in the literature for soil and sediment slurries of mixed assemblages.

Appendix C. The nepheloid sampling device on deck (A) and deployed over the side of the ship (B).

Appendix D. CTD cast profiles with depth in the water column for Gulf of Mexico/Mississippi River (15-16 March 2011) stations MSY-1 (A) and MSY-3 (B), and for Charleston Harbor system (25-26 June 2011) stations 0 (C), M (D), 6A (E), E (F), 17 (G) and A3 (H).

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Microbial Assemblage Type (Aqueous)	TNT Mineralization		Incorporation Efficiency (%)	Bacterial Metabolism (µg C L^{-1} d^{-1})	Reference
	Rate (µg C L^{-1} d^{-1})	% of added TNT			
wastewater	20 - 140	0.7	97	1.88 g L^{-1}	Kroger et al. 2004
Phanerochaete (fungi)	18	10	ND	ND	Spiker et al. 1992
Phanerochaete (fungi, lignolytic conditions)	27	30	ND	ND	Michels and Gottschalk 1994
Actinomyces (soil isolates)	33	2	96	ND	Pasti-Grigsby et al. 1996
Klebsiella (TNT enriched sludge)	41	6	99	500 (mg protein L^{-1})	Kim et al. 2002
Phanerochaete (fungi)	65	30	ND	ND	Fernando et al. 1990
Rhizobia (soil isolate)	89	< 2	ND	ND	Labidi et al. 2001
Irpex (fungi)	198	30	2	ND	Kim and Song 2003
Pseudomonas (TNT impacted soil)	< 1000	< 2	98	ND	Oh et al. 2003
Chesapeake Bay water	3	3	90	ND	Montgomery et al. (2008)
Pearl Harbor water	5	3	97	1.7 – 3.4	Montgomery et al. (2008)
Kahana Bay water	0.14 – 0.8	3	79 - 100	1.7 – 6.8	Montgomery et al. (2008)
Kahana Bay water	0.35 - 3	3	93 - 100	24 – 71	Montgomery et al. (2008)

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Appendix B. TNT mineralization (as a rate, µg kg^{-1} d^{-1}; % of added; and % d^{-1}) and incorporation efficiency (%) calculated from data provided in the literature for soil and sediment slurries of mixed assemblages.

Microbial Assemblage Type (Slurry)	TNT Mineralization		Incorporation Efficiency (%)	Bacterial Metabolism (µg C kg^{-1} d^{-1})	Reference
	Rate (µg kg^{-1} d^{-1})	% of added TNT			
soil	1	8	84-92	190 (nm CO_2 g^{-1} d^{-1})	Bradley et al. (1994)
freshwater sediment	3	0.5	99.5	10^5 (CFU d^{-1})	Wilstrom et al. (2000)
soil	20	0.5	ND	ND	Funk et al. (1993)
soil	30	8	92	ND	Gunnison et al. (1999)
soil	330	0.3	95	ND	Shen et al. (1998)
TNT impacted soil	400	30	66	5×10^6 (CFU d^{-1})	Robertson et al. (2005)
TNT impacted soil	12500	23.5	49	1×10^6 (CFU d^{-1})	Clark and Boopathy (2009)
Chesapeake Bay sediment	13 - 270	3	ND	1 - 41	Montgomery et al. (2011)
Pearl Harbor sediment	2 - 47	3	ND	0.8 - 18	Montgomery et al. (2011)
Chesapeake Bay sediment	13 - 327	3	ND	13 - 327	Montgomery et al. (2011)
San Francisco Bay sediment	1.8 - 3	3	ND	15 - 62	Montgomery et al. (2011)
Chesapeake Bay sediment	4 - 62	3	ND	4 - 62	Montgomery et al. (2011)
Kahana Bay sediment	9 - 50	3	ND	42 - 122	Montgomery et al. (2011)

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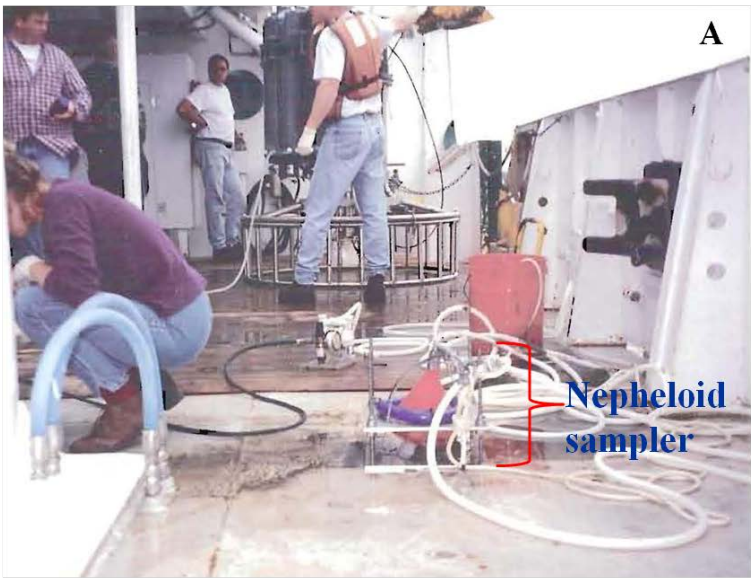
Appendix C. The nepheloid sampling device on deck (A) and deployed over the side of the ship (B).



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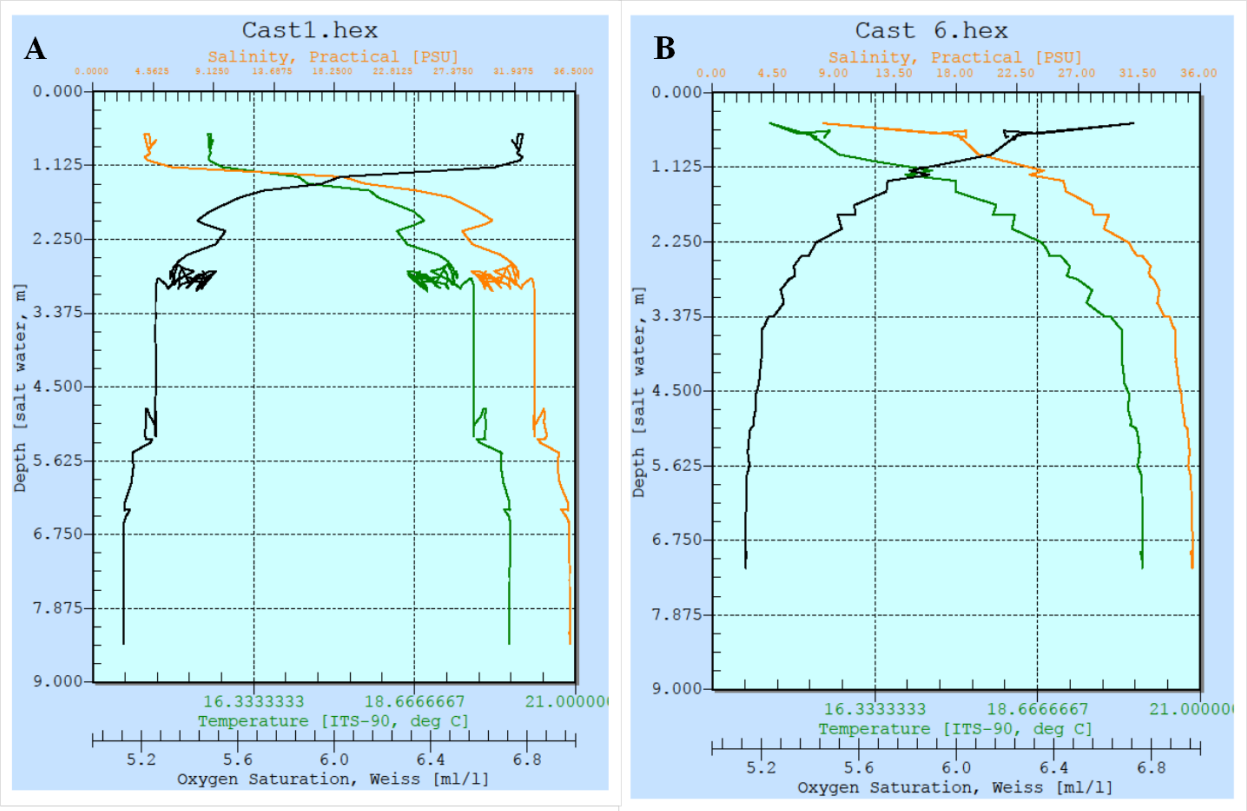

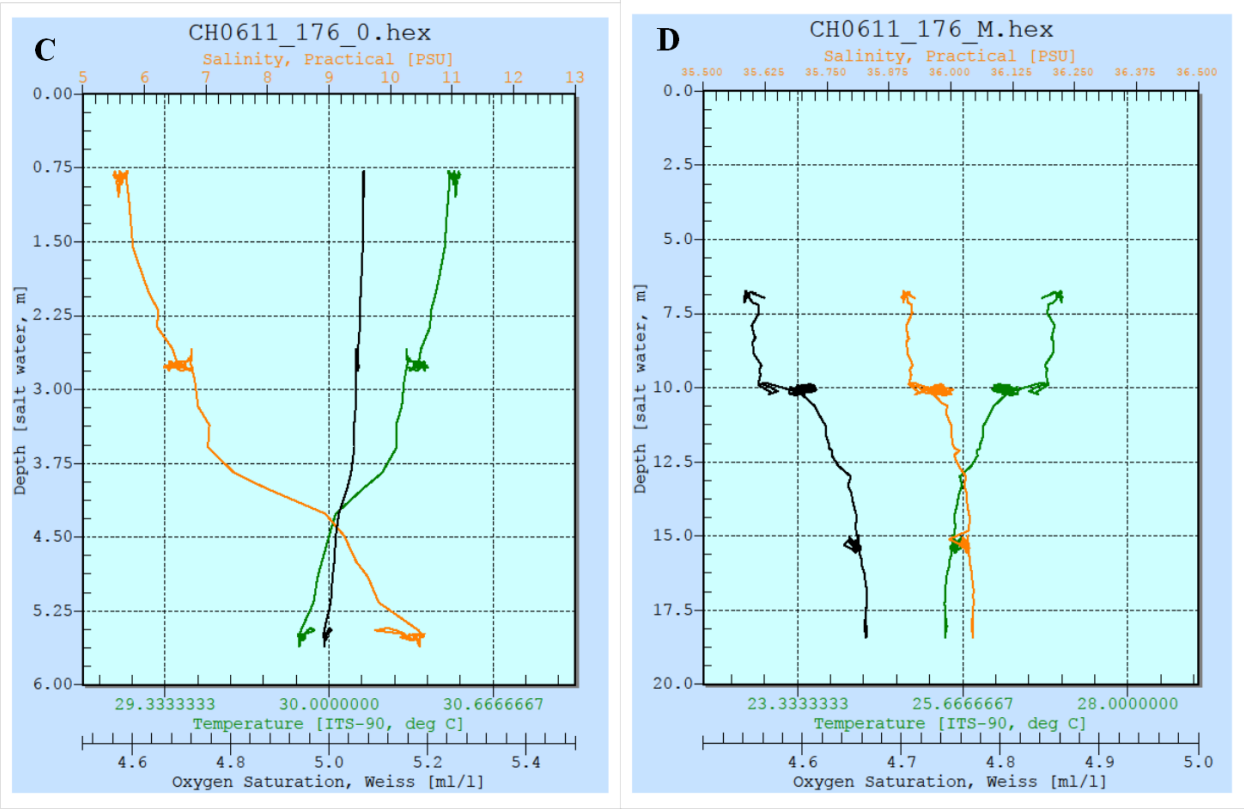
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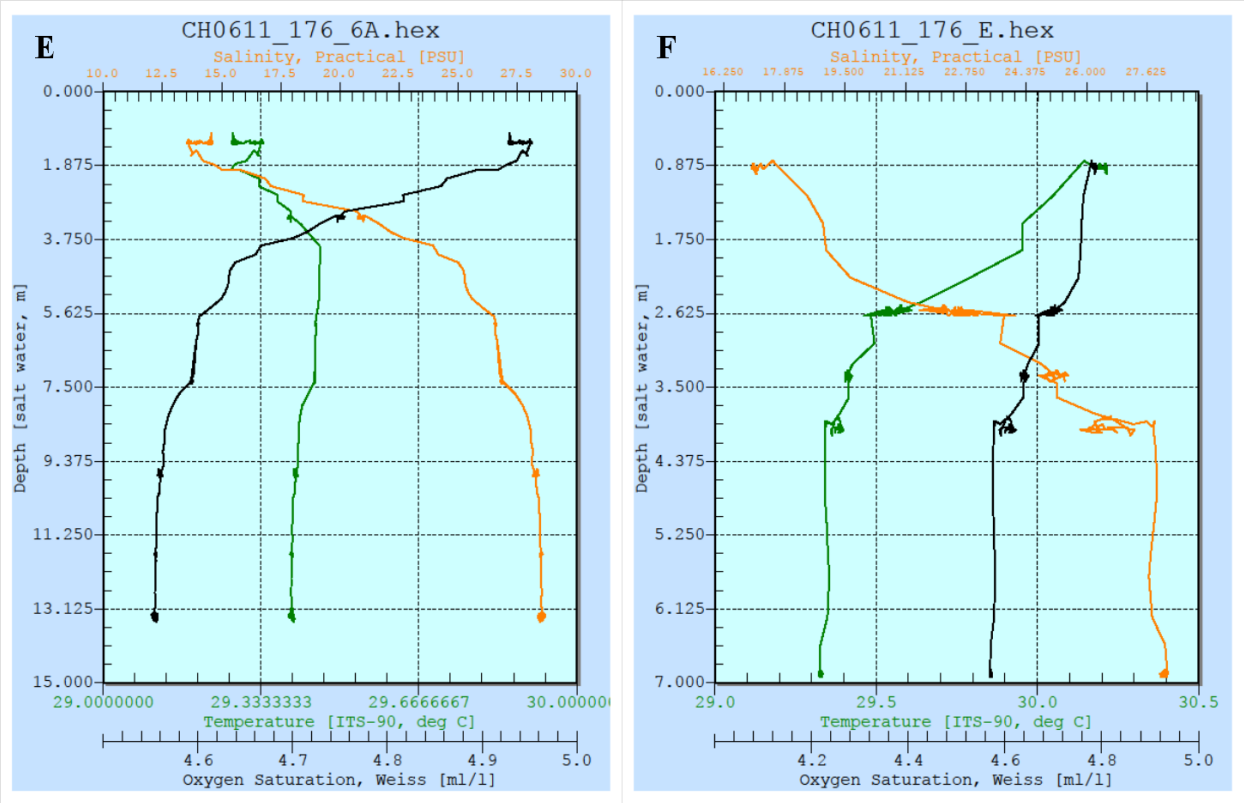
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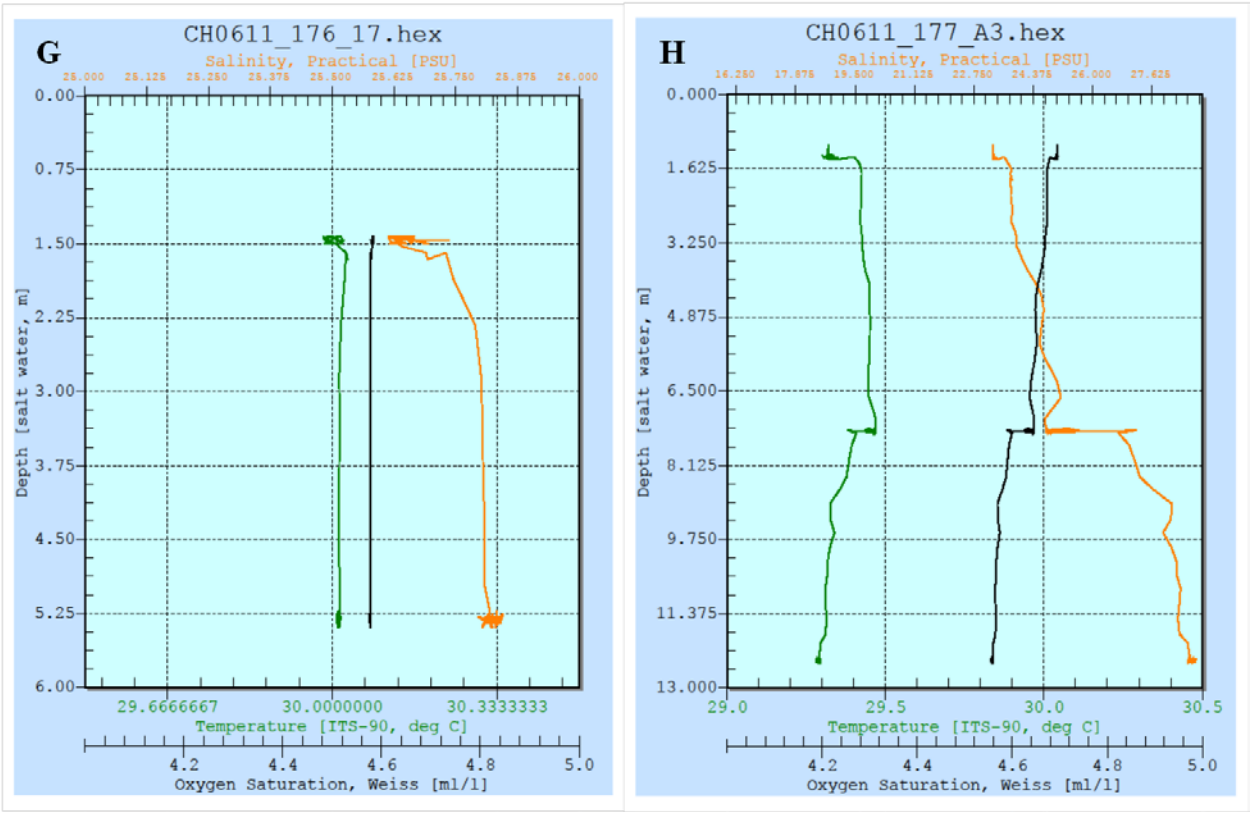
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